Saskatchewan
Agriculture
and Food

ADF

AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

20020230

DEVELOPING BRASSICA NAPUS GERMPLASM OPTIMIZED AS A MAJOR CONSTITUENT OF AQUACULTURE FEED FORMULATIONS

Funded by: The Agriculture Development Fund

October 2007

Prepared by: Agriculture and Agri-Food Canada

Final Report to ADF

Date	September 18, 2007
Reporting Period	September 2003 to September 2007
Principal Investigator	Abdelali Hannoufa
Project Number	20020230
Project Title	Developing *Brassica napus* germplasm optimized as a major constituent of aquaculture feed formulations

Abstract/Summary

There is increasing interest in using plant-based diets as a replacement for expensive and poorly sustainable fish meal in aquaculture feeds. Canola (*Brassica napus*) seed offers a sustainable alternative to conventional fish meal due to the good amino acid balance of its proteins, low cost compared to conventional fish meal, high availability and local production. However, in addition to its high content of antinutritional factors, *B. napus* seed also lacks the carotenoid pigment, astaxanthin. This is an expensive fish feed supplement, and therefore producing a *B. napus* seed that contains astaxanthin would be beneficial to both aquaculturalists and producers, especially when combined with the results of other research and development projects to reduce levels of antinutritional factors.

This project developed *B. napus* lines with elevated concentrations of astaxanthin in the seed. This was achieved by cloning two genes for astaxanthin biosynthesis from the petals of *Adonis aestivalis* and inserting them into *B. napus*. Several *B. napus* lines were developed that contained the genes responsible for astaxanthin synthesis and many of these lines had increased concentrations of astaxanthin in the seeds. *B. napus* lines were also modified to produce high concentrations of the astaxanthin precursor β-carotene. Other parallel research activities have resulted in reducing the levels of antinutritional factors, and efforts to combine the traits are underway.

Executive Summary

The long term objective of this research is to produce a new high volume crop type from *B. napus* optimized as a major constituent of fish feed ingredients. Demand for plant-based fish feed is expected to increase in the near future due to the continuing expansion of the aquaculture industry and the mounting concerns about availability, cost, health and environmental sustainability of conventional fish meal (Hites *et al.*, 2004, Science, **303**: 226). These factors provide an incentive for the fish feed industry to seek alternative feed sources and because *B. napus* seed is rich in high quality proteins and lipids, including

essential amino acids and fatty acids, an improved *B. napus* seed would be well suited to provide an abundant source of premium quality fish feed ingredients.

This project aimed to develop and implement technologies to enhance *B. napus* seed quality by producing the pigment astaxanthin to complement other research projects aimed at reducing levels of undesirable antinutrients. Astaxanthin is an essential supplement in fish feed that is responsible for giving salmonoids and shellfish their typical red-pink coloration, in addition to other health and nutritional benefits to the fish and the consumer. Astaxanthin accounts for up to 25% of the feed cost, and about 12% of the total rearing cost for cultured fish. Therefore, these improvements, when realized, will create a new niche market for *B. napus* seed by offering it to the aquaculture feed industry as a viable alternative to conventional fish meal ingredients.

In order to produce *B. napus* seeds that contained astaxanthin, two modifications were required. First, the genes for astaxanthin biosynthesis from the precursor β-carotene, β-carotene hydroxylase and β-carotene ketolase, were cloned from the petals of the plant *Adonis aestivalis*. The activity of the gene products were analysed and the genes themselves adapted for expression in *B. napus* seeds. Astaxanthin biosynthesis was confirmed in transgenic *B. napus* plants that were produced expressing these genes.

The second modification to *B. napus* plants was to increase β-carotene content in the seed from its normally low levels. By increasing the concentration of β-carotene in seeds, greater concentrations of astaxanthin could be produced with the appropriate biosynthetic machinery. Two approaches were used to increase β-carotene content in seeds; 1) overexpressing a gene for carotenoid synthesis, phytoene synthase that catalyzes the first committed step in carotenoid biosynthesis, and; 2) suppressing expression of lycopene epsilon-cyclase, an enzyme that diverts carotenoid precursors away from the branch in the carotenoid synthesis pathway leading to β-carotene. Utilising both of these approaches, we produced *B. napus* plants that over-accumulate β-carotene in the seed. Up to 45-fold and 180-fold increases in β-carotene content in seeds were observed in the pytoene synthease overexpressing lines and the lycopene epsilon cyclase suppressed lines, respectively.

These β-carotene enhanced lines of *B. napus* were used as a platform for transformation with the two genes, β-carotene hydroxylase (actH) and β-carotene ketolase (adketo2) and transgenic plants are currently being evaluated for their carotenoid content. In addition, other research and development research has generated *B. napus* lines with reduced levels of undesirable antinutritional factors. Combining all of these metabolic improvements is underway.

Technical Report:

Introduction and rationale

The carotenoid astaxanthin and its precursor canthaxanthin are necessary for fish health and impart the pink/red colour associated with salmon flesh. The marketability of salmon is related to flesh colour and, as oilseeds typically lack canthaxanthin and astaxanthin, aquaculture feed formulations that have a high

content of oilseed meal are supplemented with these compounds.

Supplementation is expensive and comprising up to 25% of the total feed cost. As approximately 50% of salmon aquaculture costs go into feed, finding ways to avoid carotenoid supplementation will reduce input costs for fish farmers. Furthermore, as oilseed-based protein is used to reduce the amount of imported fish meal used in feed formulations, the greater use of oilseed meal will improve the sustainability of fish feed production. Finally, the amount of organic contaminants present in farmed salmon is significantly higher than wild salmon (Hites *et al.*, 2003, Science **303**:226) and as a consequence, may reduce consumption levels of the aquaculture raised product. Replacing fish meal with *B. napus* meal reduces the amounts of PCBs present in aquaculture raised fish (Drew *et al.*, 2007, Aquaculture, **267**, 260), improving the safety of the product.

With modifications, *B. napus* seeds could be an ideal source of both protein and carotenoids in fish feeds. *B. napus* seeds contain β-carotene, a precursor of canthaxanthin and astaxanthin. *B. napus* seed meal is currently used in fish feeds because of its low cost (compared to fish-meal derrived protein), good amino acid balance, sustainable production and wide availability (Maenz *et al.*, 2003, Maenz *et al.*, 2003 in Proc. 11th IRC, pp. 1234-1236). Drawbacks of using *B. napus* meal for fish meal are the presence of antinutritional substances such as lignin, sinapine and phytate and the low concentration of carotenoids such as β-carotene and astaxanthin.

The potential for astaxanthin-containing, low antinutritional content *B. napus* seed in the aquaculture feed market is large. The annual value of the 110,000 tonne salmon market in Canada alone is $385 m. If 25% of feed is comprised of *B. napus* meal and 1.2 kg dry feed is required to produce 1 kg of salmon, then 33,000 T of meal would be needed per year. Yields and the number of operations on both coasts are expected to increase substancially over the next 15 years. As Canada is the fourth largest farmed salmon producer, behind Norway, Chile and the United Kingdom, substantial amounts of *B. napus* meal could also be exported. In addition, the large shrimp aquaculture industry would benefit from astaxanthin-containing *B. napus* meal as the pink colour in crustaceans is also derived from this carotenoid.

There were several goals in this research:

1) Increasing β-carotene content in *B. napus* seeds
2) Modifying *B. napus* to convert β-carotene to astaxanthin
3) Investigating the potential of moving the astaxanthin synthesis machinery into *B. napus* lines with low concentrations of antinutritional substances

Adonis aestivalis (common name: Summer Pheasant's Eye) is one of the few higher plants that accumulate astaxanthin. This plant converts β-carotene to astaxanthin by one of two pathways involving the enzymes β-carotene hydroxylase (*actH*, also called CrtH1) and β-carotene ketolase (*actK*). The first route involves the hydroxylation of β-carotene to zeaxanthin by *actH* followed by the oxidation of zeaxanthin to astaxanthin by *actK* (Fig 1). The second involves the oxidation of β-carotene to canthaxanthin by *actK*, which is then hydroxylated to

produce astaxanthin via *actH* (Mann *et al.*, 2000, Nat. Biotech. **18**: 888). Although *B. napus* seeds do not contain astaxanthin, they do contain the precursor molecule, β-carotene, at low levels.

Inserting the genes for *actH* and *actK* from *A. aestivalis* and into *B. napus* plants should allow the conversion of β-carotene to astaxanthin. Although *actH* and *actK* have been cloned from bacteria and algae, the orthologous genes in plants had not been isolated from higher plants. We thought it was important to utilise genes from higher plants for expression in *B. napus* to optimise for gene expression and enzyme activity. Therefore, the first part of this project was to clone these genes from *A. aestivalis*.

Yields of β-carotene in *B. napus* seeds are low (approximately 0.5 µg/g dry weight). A higher concentration of β-carotene in *B. napus* seeds that contain *actH* and *actK* is required in order to produce sufficient concentrations of astaxanthin. Increasing carotenoid concentrations in plants has been demonstrated before, usually by overexpression of bacterial carotenoid biosynthesis genes (e.g. Shewmaker *et al.*, 1999, Plant J. **20**: 401). We chose to use plant-derived sequences to alter carotenoid concentrations in seeds to avoid unforeseen problems with expressing bacterial genes in plant cells and to optimise enzyme activity.

Two strategies based on altering the expression of key genes in carotenoid biosynthesis were adopted to enhance carotenoid content in the seed;

1) We overexpressed the phytoene synthase (PSY) of *Arabidopsis thaliana* in a seed-specific manner. Previous work showed that plants overexpressing PSY, the first step in the biosynthesis pathway of carotenoids, produced higher concentrations of these compounds (Lindgren *et al.*, 2003, Plant Physiol. **132**: 779).
2) We downregulated the expression of *B. napus* lycopene ε-cyclase (ε-CYC) by RNAi. This enzyme functions at the branch point in the carotenoid pathway (Fig 1). We hypothesised that higher concentrations of β-carotene and lower concentrations of lutein would be produced by reducing ε-CYC activity.

Although *B. napus* seed meal is widely used as a protein source in animal feeds, the high concentrations of antinutritional compounds reduce the nutritional value somewhat. Sinapine, which is unique to crucifers, adversely affects diet palatability and digestibility. Levels range from 0.7% to 3%, with about 90% of it present in the embryo fraction (Larsen *et al.*, 1983, in Proc 6th IRC, pp1577). Dietary fibre represents about a third of the canola meal remaining after oil extraction. High fibre content can have adverse effects on diet digestibility, feed efficiency, and also reduces oil and protein yields (Hilton *et al.*, 1983, Can. J. Fish Auat. Sci. **40**: 81). Phytate, which ranges from 2.0-4.0% in the seed, has both nutritional and environmental drawbacks (Hurrell 2003, J. Nutr., **33**: 2973S). It reduces the phosphorus directly available to fish, while at the same time causing release of unassimilated phosphate into the environment where it causes major problems, especially in fresh water aquaculture. Therefore, we have been conducting research (funded through other sources) aimed at

reducing the levels of these antinutritional factors. This research is developing *B. napus* lines with low concentrations of these antinutritional substances in the seeds to improve their nutritional qualities. Inserting the genes for the production of high concentrations of astaxanthin into *B. napus* seeds containing low levels of antinutritional substances will result in a product with high value in the aquaculture industry. Combining traits could be achieved by conventional breeding, the development of a hybrid seed system or by genetically modifying directly the low-antinutrient content lines.

Objectives

1) Clone two genes involved in astaxanthin biosynthesis, β-carotene hydroxylase and β-carotene ketolase from *A. aestivalis*
2) Engineer β-carotene hydroxylase and β-carotene ketolase for expression in developing *B. napus* seeds
3) Produce transgenic *B. napus* containing higher concentrations of β-carotene via PSY overexpression or ε-CYC silencing
4) Produce transgenic *B. napus* expressing β-carotene hydroxylase and β-carotene ketolase with the aim of engineering astaxanthin biosynthesis

Objective 1) Clone two genes involved in astaxanthin biosynthesis, β-carotene hydroxylase and β-carotene ketolase from, *A. aestivalis*

Conserved regions in β-carotene hydroxylase and β-carotene ketolase from various organisms were identified and degenerate primers were designed to PCR amplify these from *A. aestivalis*. The amplified fragments were analyzed by DNA sequencing. To isolate the full length cDNAs 3'- and 5'- Rapid Amplification of cDNA Ends (RACE) was used on RNA from *A. aestivalis* flower petals. A full-length cDNA of *actH* (1187bp) was cloned. It encodes a predicted protein of 309 amino acids with a molecular weight of ~35 kDa, a pI of 9.15, a 59 amino acid chloroplast peptide targeting sequence (identified using Chloroplast v1.1 (http://www.cbs.dtu.dk/services/ChloroP/)) and ~70% amino acid identity with other plant β-carotene hydroxylases.

Detailed genetic and functional characterization of actH was performed. These findings are summarized here and were presented in detail in a scientific publication (Yu *et al.*, 2007, Planta **226**:181–192). Genomic analysis of *actH* revealed that it is a member of a multigene family in *A. aestivus*. Astaxanthin is found in *A. aestivalis* petals, so it was expected that mRNAs for both β-carotene hydroxylase and β-carotene ketolase would be expressed in this organ. The expression pattern of these two genes were confirmed in the petals of *A. aestivalis*, as well as the stems and roots by Northern blotting (Fig. 2).

The *actH* cDNA was inserted into pBluescript and used to transform *E.coli* cells containing genes for β-carotene synthesis (Misawa *et al.*, 1995, Biochem. BioPhys Res Comm. **209**: 867-876). Strains containing *actH* produced zeaxanthin and cryptoxanthin in addition to the β-carotene observed in strains lacking the gene (Fig. 3). This demonstrated that the cloned *actH* enzyme was able to perform the first step in the formation of astaxanthin.

The high content of cryptoxanthin in the *E. coli* strains suggested that the *actH* enzyme was not functioning optimally in bacterial cells. The gene was modified for expression in *A. thaliana* seeds to determine if the enzyme functioned properly in plant cells. The *actH* was inserted into both wild type and β-carotene hydroxylase deficient *A. thaliana* lines under control of the napin seed-specific promoter. Seeds from transgenic plants containing *A. aestivus actH* contained less β-carotene and more violaxanthin, lutein and, in some lines, cryptoxanthin than non-transformed controls (Table 1). As violaxanthin results from the epoxidation of zeathanthin, the at least three-fold greater concentration of the former in the transgenic lines, compared with untransformed lines, indicated that the *actH* enzyme functions well in *A. thaliana* seeds. That is, the *actH* enzyme hydroxylated β-carotene to zeaxanthin, which was converted to violaxanthin by the endogenous zeaxanthin epoxidase. The large increase in lutein content, especially in the β-carotene hydroxylase mutant, was probably due to hydroxylation of the β-ring of α -carotene, the precursor of lutein, by *actH*. This data suggests that the *actH* acts as expected in a plant cell background.

During the course of this work other researchers isolated an *actK* gene from *A. aestivalis* (Cunningham and Gantt, 2005. Plant J. **41**:478). We cloned a cDNA of this gene (*Adketo2*) using RT-PCR and used it in subsequent work.

Outcome: Complete

Objective 2) Modify β-carotene hydroxylase and β-carotene ketolase for expression in developing *B. napus* seeds

The cloned *actH* and *adketo2* were inserted between a promoter and a 3' *nos* termination sequence. β-carotene hydroxylase (actH, p710-433) was placed under the control of the napin seed-storage protein promoter whilst β-carotene ketolase (adketo2. p710-438) was inserted downstream of the constitutive tCUP promoter (Fig 4). A construct for the co-expression of the two genes was also generated (p710-440). Each of these modified genes was inserted into a plant expression vector (Yu *et al.*, 2007, Planta, **226**, 181) and introduced into *B. napus* doubled haploid line DH12075 via *Agrobacterium* mediated transformation (Moloney *et al.*, 1989, Plant Cell Rep. **8**:238).

Lines were made containing either one of the genes or both (Fig. 4). Lines with single genes were constructed in case the production of high concentrations of astaxanthin in the seeds had a detrimental affect on seed development, survival or germination. As both genes are required to produce astaxanthin, expression of a single enzyme from the pathway would not result in the production of astaxanthin. Conventional crossing would allow both genes to be expressed in seeds. In addition, once commercialisation of high astaxanthin varieties was started, this strategy would facilitate the production of hybrid parent lines. The construct containing both genes was made to quickly determine if this strategy would work and to allow comparisons with the single-gene containing lines to be made. Both conventional brown seeded (DH12075) and yellow seeded (YN01-429) *B. napus* germplasms were used for transformation by astaxanthin biosynthesis genes, *actH* and *adketo2*.

1. Characterization of *B. napus* lines expressing *actH*
A total of 100 *B. napus* lines expressing the *actH* gene were generated and their seeds analyzed for carotenoid content. Seeds of at least eight trangenic lines showed an overall increase in the levels of different carotenoids, especially β-carotene and lutein (Table 2).

2. Characterization of *B. napus* lines expressing *adketo2*
About 80 transgenic *B. napus* lines expressing *adketo2* were generated and their seeds analyzed for carotenoid contents. At least seven lines showed significant increases in the levels of β-carotene and lutein. In lines derived from the *B. napus* DH12075, we could also detect small quantities of astaxanthin (Table 3). This could be due to the expression and function of the endogenous β-carotene hydroxylase. The absence of staxanthin from lines derived from the yellow seeded YN01-429 may indicate absence or low expression of β-carotene hydroxylase in this germplasm.

3. Characterization of *B. napus* lines expressing *actH and adketo2*
Of the 80 transgenic *B. napus* lines expressing both *actH* and *adketo2*, 10 showed increased levels of β-carotene and lutein, and six had astaxanthin (Fig. 5, Table 4). Transgenic lines derived brom both parental lines, DH12075 and YN01-429, had astaxanthin in the seed at about 2 µg/g FW. This indicates that both actH and adketo2 were expressed and were functional in both *B. napus* germplasms.

Outcome: Complete

Objective 3) Produce transgenic *B. napus* containing higher concentrations of β-carotene

Overexpression of phytoene synthase

Previous work showed that overexpression of phytoene synthase (PSY) in *Arabidopsis* seeds led to an increase in total carotenoids, including β-carotene (Lindgren *et al.*, 2003, *Plant Physiol.* **132**: 779). We set out to determine to what extent repeating the experiment in *B. napus* would have on β-carotene content. The *Arabidopsis* PSY (*AtPSY*) cDNA was isolated from seed mRNA using RT-PCR. This gene was modified for expression in *B. napus* by placing the whole cDNA, including the 5' plastid targeting signal peptide sequence, under the control of the constitutive CaMV 35S promoter or the seed-specific napin promoter. Both constructs were inserted into *B. napus* via *Agrobacterium*-mediated transformation and several independant lines for each construct were recovered. The presence of the modified *AtPSY* gene in the recovered transgenic *B. napus* plants was confirmed by PCR and Southern blotting, and the carotenoid profile was determined by HPLC. The *AtPSY* lines had an up to 45-fold increase in β-carotene content in the seeds (Fig. 6, Table 5), compared with parental type plants.

Silencing of lycopene ε-cyclase

Reducing the expression of lycopene ε-cyclase (ε -CYC) was another strategy we followed to increase β-carotene levels in seeds. ε -CYC functions at the branch point in the carotenoid biosynthesis pathway (Fig 1), and controls the channeling of the precursor lycopene towards the the formation of lutein and away from β-carotene. RNAi technology was used to silence (suppress) *ε -CYC* expression in *B. napus* seeds. Two *B. napus* ESTs with homology to the *A. thaliana* ε -CYC were identified from a library held at the Saskatoon Research Centre. Short sequences corresponding to the 3' and 5' ends of *ε -CYC* were amplifed using PCR for use in the RNAi constructs. Palindromic repeats from these fragments were placed on either side of the unique GUS spacer in the RNAi-vector pGSA1285 (CAMBIA, Canberra, ACT, Australia). These constructs were then inserted into *B. napus* DH12075 using *Agrobacterium* mediated transformation.

As expected, plants containing the RNAi construct had reduced levels of ε -*CYC* transcript, while levels of other genes in the pathway remained largely unchanged (Fig. 7). Seeds from plants with silenced *ε -CYC* had an up to 180-fold increase in β-carotene levels compared with parental type plants (Fig. 8, Table 6). RNAi silencing of *ε -CYC* successfully increased concentrations of the astaxanthin precursor and so could be potential targets for insertion of the *actH* and *adketo2* genes. RNAi silencing of *ε -CYC* slightly reduced the concentrations of fatty acids present in the seeds. This may have been due to the increased concentration of β-carotene in the seeds. β-carotene is a precursor of absicic acid and higher concentrations of this plant growth regulator in the seeds may have fore-shortened seed maturation. Shortened maturation times in seeds sometimes lead to reduced concentrations of fatty acids and oils. This may not be a problem in plants containing the astaxanthin-synthesis enzymes as much of the β-carotene will be converted to zeaxanthin and astaxanthin, preventing the increased synthesis of abscisic acid.

The ε -CYC silenced plants also had higher concentrations of other carotenoids including, unexpectedly, lutein. We hypothesise that silencing *ε -CYC* removed a limiting step in the carotenoid pathway, allowing a much faster rate of carotenoid synthesis. The production of lutein may have been caused by other members of the *ε -CYC* gene family or through lutein production via an undiscovered biochemical pathway.

More details about increasing β-carotene content in *B. napus* seeds by silencing *ε -CYC* are given in a manuscript that has been accepted for publication in Transgenic Research (Yu *et al.*, 2007, Transgenic Res.; Epub ahead of print).

The transgenic *B. napus* ε -CYC RNAi line BY223 with high β-carotene content was used as platform for transformation with *actH* and *adKeto2* to enhance astaxanthin production. Transgenic lines have been generated and are currently undergoing molecular analysis to assess gene expression levels and chemical analysis to determine carotenoid profiles. Once lines with astaxanthin content (at least 50 µg/g F.W.) are produced, they will be crossed to other lines containing low concentrations of antinutritional substances to produce a seed highly optimized for aquaculture feed formulations.

Outcome: Complete

Conclusions and discussion

We were successfull in generating *B. napus* lines that produce astaxanthin in the seed. When combined with the *B. napus* lines containing low concentrations of antinutritional factors, seeds from these plants will be very suitable for use in aquaculture feeds. Both producers and salmon and shrimp aquaculturalists will benefit from using these types of *B. napus* lines.

Future directions

- Move high-astaxanthin trait into low antinutritional factor lines
- Introduce quality traits into breeding lines of *B. napus*
- Introduce one of each trait into separate parent lines, which are used to produce hybrid seed containing genes from both transgenic parents
- Further studies of carotenoid biosynthesis pathway
- Feeding tests on meal, meal reconstituted with extracted oil and whole seeds
- Carcas and market analysis from salmon and shrimp fed with high-astaxanthin line *B. napus* seeds or seed meal

Publications and presentations

Peer Reviewed Publications

1. Yu B., Lydiate D.J., Young L.W., Schäfer U.A. and Hannoufa A. (2007) Enhancing the carotenoid content of *Brassica napus* seeds by downregulating lycopene epsilon cyclase. Transgenic Res. (Epub ahead of print)
2. Yu B., Lydiate D.J., Schäfer U.A. and Hannoufa A. (2007) Characterization of a β-carotene hydroxylase of *Adonis aestivalis* and its expression in *Arabidopsis thaliana*. Planta **226**:181–192

Patent Application

Hannoufa A., Lydiate D.J., Yu B., Schäfer U.A. (2007) Altering carotenoid profiles in plants. US Patent Application # 11/709,571

Oral presentations

1. Hannoufa, A. (2004) Optimizing *Brassica napus* for Aquaculture Feed Formulations. Presented at the "*Applying Genomics to B. napus Improvement Workshop*" in Saskatoon (December 9, 2004)

2. Hannoufa A. (2007) Modification of Cruciferous Secondary Metabolism; Progress towards Enhancing Seed Quality. Presented at the 9th Annual General Meeting of AAFC's Crop Genomics Initiative, Penticton, BC (Sept 12-13, 2007).

Poster presentations

1. Yu B., Lydiate D., Schäfer U., Gropp G., and Hannoufa A. (2004) Molecular cloning and characterization of ß-carotene hydroxylase and ß-carotene ketolase from *Adonis aestivalis*. *Second Canadian Plant Genomics Workshop*, Québec, QC

2. Yu B., Lydiate D., Schäfer U. and Hannoufa A. (2005) Molecular and Functional Characterization of β-Carotene Hydroxylase of *Adonis aestivalis*. *3rd Canadian Plant Genomics Workshop*, Saskatoon, SK

3. Yu B., Schäfer U., Lydiate D., and Hannoufa A. (2006) Molecular and functional characterization of ß-carotene hydroxylase of *Adonis aestivalis*. *4th Canadian Plant Genomics Workshop*, Ottawa, ON

4. Yu B., Lydiate D., Schäfer U., Hannoufa A. (2006) Cloning and functional characterization of β-carotene hydroxylase of *Adonis aestivalis*. *8th ISPMB*, Adelaide, Australia

5. Yu B., Lydiate D., Schäfer U., Hannoufa A. (2006) A novel β-carotene hydroxylase from *Adonis aestivalis*; molecular cloning and functional characterization. *FESPB 2006 Congress*, Lyon, France

6. Yu B., Lydiate D., Young L.W., Schäfer U., Hannoufa A. (2007) Enhancing carotenoid content of *Brassica napus* seeds by downregulating lycopene epsilon cyclase. *12th International Rapeseed Congress*, Wuhan, China and Plant Canada 2007, Saskatoon, SK

Benefits to stakeholders from the project

The introduction of Brassica oilseeds as a major component in fish feed would generate a new large-scale value-added market for a new crop agronomically identical to *B. napus*. *B. napus* (canola) is well suited for production in Western Canada. Developing a modified *B. napus* that accumulates astaxanthin, combined with the products of other research and development projects to reduce antinutritional factors in the seed, would constitute a major advance towards improving *B. napus* seed as an excellent source of ingredients for formulation of premium quality fish feed. The products of the proposed research will provide a strong financial incentive for the aquaculture industry to adopt feed formulations based on *B. napus* oilseeds by supplying the pigment astaxanthin as an integral component of the meal. The fish feed market is potentially very large and, if these improvements are realized, will require cultivation on millions of acres annually. Supplying an alternative to *B. napus* should reduce the acreage of conventional *B. napus* and assist in stabilizing *B. napus* commodity prices. Ensuring continued large-scale production of *Brassica* oilseed crops in the Canadian prairies will help to maintain

crop diversification and assist in the economic and environmental sustainability of the agricultural system.

Personnel

Name	Position	Source	Time (FTE)
Bianyun Yu	PostDoctoral Fellow	ADF	100% (09/03 to 09/06)
S. Chellama	Biologist	ADF	100% (04/07 to 09/07)
A. Hannoufa	Research Scientist	AAFC	20% (09/03 to 09/07)
D. Lydiate	Research Scientist	AAFC	10% (09/03 to 09/07)
R. Schäfer & vice	EG 04/05 Technician	AAFC	10% (09/03 to 09/07)

Project Developed Materials

- *Adonis aestivalis* petal cDNA library
- A full length, annotated cDNA of β-carotene hydroxylase (GeneBank accession number EF120636)
- Characterisation of *actH/CrtH1* gene and enzyme
- *E. coli* strain expressing *actH*
- Wildtype and mutant *Arabidopsis* lines expressing *actH*
- Several cDNA fragments with similarity to other β-carotene hydroxylases and β-carotene ketolases
- β-carotene hydroxylase and β-carotene ketolase cDNAs modified for expression in *B. napus*
- Plasmids and strains of *Agrobacterium* carrying one or both modified astaxanthin-synthesis genes
- Two types of transgenic *B. napus* plants with higher carotene concentrations in the seeds
- plasmids and strains of *Agrobacterium* containing genes useful for increasing carotenoid concentrations in plants (PSY cDNA and ε-CYC RNAi)
- cDNA of *B. napus* lycopene ε-cyclase
- high β-carotene content lines containing astaxanthin-synthesis genes
- A collection of at least 2000 EST sequences from the *Adonis aestivalis* flower petals cDNA

Acknowledgements

ADF support was acknowledged when public and private sector representatives visited AAFC-SRC. ADF was acknowledged in oral presentations at the "Applying Genomics to Canola Improvement Workshop" in Saskatoon (December 9, 2004) and the 9th Annual General Meeting of AAFC's Crop Genomics Initaiative (September 12-13, 2007), and in an oral presentation to a delegation from the Chilean Department of Agriculture (April 2007). ADF was acknowledged in poster presentations at the 12th International Rapeseed Congress (Wuhan, China), Plant Canada 2007 Meeting (Saskatoon), 8th ISPMB

Meeting, the FESPB 2006 Congress, the the 2nd, 3rd and 4th Canadian Plant Genomics Workshops. ADF was also acknowledged in two publications (Yu *et al.*, Planta **226**:181–192 and Yu *et al.*, Trans. Res. Transgenic Res., Epub ahead of print, and in another manuscript that is currently in preparation.

Project expenses

See attached financial report from AAFC-Saskatoon.

References

Cunningham F.X. Jr., Gantt E. (2005) A study in scarlet: enzymes of ketocarotenoid biosynthesis in the flowers of Adonis aestivalis Plant J. **41**:478-492

Drew M.D., Ogunkoya A.E., Janz D.M., Van Kessel A.G.(2007) Dietary influence of replacing fish meal and oil with canola protein concentrate and vegetable oils on growth performance, fatty acid composition and organochlorine residues in rainbow trout (Oncorhynchus mykiss). Aquaculture, **267**: 260–268

Hilton J.W., Atkinson J.L. and Slinger S.L. (1983) Effect of increased dietary fibre on the growth of rainbow trout (*Salmo gairdneri*) Can. J. Fish Aquat. Sci., **40**: 81-85

Hites R.A., Foran J.A., Carpenter D.O., Hamilton M.C., Knuth, B.A., Schwager S.J. (2004) Global Assessment of Organic Contaminants in Farmed Salmon. Science, **303**: 226-229

Hurrell R.F. (2003) Influence of Vegetable Protein Sources on Trace Element and Mineral Bioavailability J. Nutr., **33**: 2973S

Larsen *et al* (1983) In *Proceedings of the Sixth International Rapeseed Conference*, Paris, France, pp. 1577

Lindgren L.O., Stålberg K.G., Höglund A.S. (2003) Seed-Specific Overexpression of an Endogenous Arabidopsis Phytoene Synthase Gene Results in Delayed Germination and Increased Levels of Carotenoids, Chlorophyll, and Abscisic Acid Plant Physiol. **132**: 779-785

Maenz D.D., Newkirk R.W., Classen H.L. Thiessen D.L., Drew M.D. and Lahti T.L. (2003) The nutritional value of rapeseed protein concentrate (MCN BioProducts Inc.-CanPro-IP65) for rainbow trout. Proc. 11th International Rapeseed Congress, pp. 1234-1236

Mann V., Harker M., Pecker I., Hirschberg J.(2000) Metabolic engineering of astaxanthin production in tobacco flowers Nat. Biotech. **18**: 888-892

Misawa N., Kajiwara S., Kondo K., Yokoyama A., Satomi Y., Saito T., Miki W., Ohtani T. (1995), Canthaxanthin Biosynthesis by the conversion of methylene to

keto groups ina hydrocarbon B-Carotene by a single gene Biochem. BioPhys Res Comm. **209**: 867-876

Moloney M.M., Walker J.M., Sharma K.K. (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep. **8**: 238-242

Shewmaker C.K., Sheehy J.A. Daley, M. Colburn, S., Ke, D.Y., (1999) Seed-specific overexpressionof phytoene synthases: increase in carotenoids and other metabolic effects Plant J. **20**: 401-412

Yu B., Lydiate D.J., Young L.W., Schäfer U.A. and Hannoufa A. (2007) Enhancing the carotenoid content of Brassica napus seeds by downregulating lycopene epsilon cyclase. Trans. Res. (Epub ahead of print)

Yu B., Lydiate D.J., Schäfer U.A. and Hannoufa A. (2007) Characterization of a β-carotene hydroxylase of *Adonis aestivalis* and its expression in Arabidopsis thaliana. Planta **226**:181–192

	violaxanthin µg/g dw	lutein µg/g dw	zeaxanthin µg/g dw	β-cryptoxanthin µg/g dw	β-carotene µg/g dw
wt	0.694±0.056	13.380±0.729	0.955±0.051	UD	1.890±0.504
wt+actH					
BY275	3.186±0.544	13.413±3.354	0.776±0.193	UD	1.009±0.188
BY280	5.47±0.342	21.631±0.941	1.162±0.036	UD	0.422±0.033
BY281	6.162±0.416	20.684±0.623	1.067±0.033	UD	0.4±0.007
BY284	2.939±0.199	12.678±1.367	0.642±0.100	UD	0.718±0.081
BY287	7.572±0.284	22.016±0.483	1.077±0.011	UD	0.591±0.068
BY293	5.611±0.216	21.49±0.597	0.895±0.034	0.098±0.002	1.491±0.142
BY294	3.975±0.12	19.306±0.95	0.883±0.016	UD	1.015±0.074
b1b2 mutant	0.881±0.036	26.214±2.690	0.711±0.082	0.153±0.014	8.238±1.019
b1b2 mutant +actH					
BY317	24.741±2.37	42.523±2.275	0.727±0.023	UD	2.473±0.439
BY330	5.522±1.295	25.912±4.857	0.544±0.048	0.139±0.043	6.41±0.081
BY334	8.713±1.092	34.809±2.493	0.611±0.039	0.156±0.006	8.942±0.88
BY341	11.005±0.548	33.705±2.331	0.526±0.061	0.216±0.075	5.78±0.435
BY342	20.62±0.688	45.199±0.709	0.731±0.025	UD	3.099±0.228
BY346	15.111±1.103	36.464±1.591	0.584±0.037	0.164±0.013	3.262±0.11
BY347	3.871±0.163	30.08±1.882	0.616±0.014	0.148±0.015	6.985±0.515

Table 1: Concentrations of carotenoids in *Arabidopsis thailana* seeds from select lines transformed with *actH* gene. UD, undetectable; dw, dry weight; Each value is the mean result from triplicate ± SD

Plant line	Violaxanthin	Lutein	Zeaxanthin	Astaxanthin	β-carotene
DH12075	UD	0.69±0.27	UD	UD	0.31±0.23
DE1179	0.26±0.07	6.34±1.28	0.22±0.05	UD	3.75±0.91
DE1181	UD	2.89±1.07	UD	UD	0.85±0.30
DE1184	UD	1.62±0.23	UD	UD	0.74±0.31
DE1298	UD	2.81±0.13	UD	UD	1.07±0.15
DE1305	0.18±0.03	3.05±0.52	0.14±0.01	UD	2.18±0.77
DE1307	0.08±0.03	1.16±0.03	UD	UD	0.20±0.02
DE1315	UD	1.61±0.24	UD	UD	0.18±0.05
DE1321	UD	1.14±0.05	UD	UD	0.06±0.01
DE1374	UD	1.67	UD	UD	1.05
DE1343	UD	3.86	UD	UD	2.77
DE1299	UD	3.38	0.09	UD	0.67
DE1309	0.17	7.95	0.28	UD	0.46
YN01-429	UD	1.26±0.26	UD	UD	0.14±0.02
DE1522	0.09±0.04	2.52±0.51	0.09±0.04	UD	2.20±0.38
DE1536	UD	1.77±0.56	UD	UD	1.40±0.90
DE1640	0	1.58±0.23	UD	UD	0.78±0.10
DE1527	0.18±0.09	5.82±0.37	UD	UD	0.78±0.13
DE1523	0.04	4.21	UD	UD	0.62
DE1518	0.57	9.33	UD	UD	0.98
DE1638	UD	2.32	UD	UD	1.47
DE1644	0.12	2.55	0.13	UD	1.79

Table 2 Carotenoid concentrations in transgenic *Brassica* seeds with construct p710-433

Values expressed as μg/g FW; UD, undetectable; FW, fresh weight

Plant line	Violaxanthin	Lutein	Zeaxanthin	Astaxanthin	β-carotene
DH12075	UD	0.69±0.27	UD	UD	0.31±0.23
DE1368	UD	2.37±0.28	UD	2.01±0.15	1.82±0.833
DE1369	UD	1.21±0.15	UD	1.72±0.01	0.71±0.107
DE1371	UD	1.90±0.64	UD	1.96±0.09	1.64±0.38
DE1372	UD	1.40±0.37	UD	1.93±0.10	0.47±0.21
DE1178	UD	3.43	0.33	2.32	1.92
DE1200	0.15	9.11	UD	2.31	2.62
DE1210	UD	2.28	UD	1.83	3.49
DE1216	0.25	11.09	1.65	2.97	4.74
DE1318	UD	1.12	UD	UD	0.73
DE1364	0.09	2.47	UD	2.26	7.09
DE1373	UD	3.75	0.15	2.7	4.07
YN01-429	UD	1.26±0.26	UD	UD	0.14±0.02
DE1551	UD	2.15±0.31	UD	UD	1.02±0.15
DE1660	UD	2.36±0.45	UD	UD	0.47±0.07
DE1541	0.09	6.17	UD	1.83	UD
DE1544	UD	1.35	UD	UD	UD
DE1554	UD	3.28	0.16	1.64	UD
DE1558	0.09	3.72	UD	1.86	UD

Table 3. Carotenoid concentrations in transgenic *Brassica* seeds with construct p710-438
Values expressed as µg/g FW; UD, undetectable; FW, fresh weight

Plant line	Violaxanthin	Lutein	Zeaxanthin	Astaxanthin	β-carotene
DH12075	UD	0.69±0.27	UD	UD	0.31±0.23
DE1212	UD	2.87	UD	1.87	4.96
DE1236	UD	4.81	UD	UD	0.72
DE1247	UD	5.13	UD	2.11	1.48
DE1323	UD	4.12	UD	1.81	0.55
DE1339	UD	3.00	UD	2.46	2.41
DE1340	UD	2.84	UD	UD	0.75
YN01-429	UD	1.26±0.26	UD	UD	0.14±0.02
DE1493	0.07±0.03	2.13±0.33	UD	UD	1.09±0.47
DE1495	UD	3.50±1.22	UD	UD	0.22±0.10
DE1499	0.21±0.01	5.53±0.21	UD	UD	0.87±0.065
DE1664	UD	11.48±2.01	UD	UD	0.97±0.62
DE1671	0.65±0.09	6.52±0.29	UD	1.96±0.05	0.79±0.09
DE1675	0.17±0.01	5.07±0.81	0.09±0.01	1.77±0.02	6.59±0.88
DE1677	0.09±0.04	3.91±0.27	UD	UD	0.57±0.03
DE1672	0.22	6.86	0.103	UD	UD
DE1678	1.33	17.65	0.214	5.41	UD

Table 4. Carotenoid concentrations in transgenic *Brassica* seeds with construct p710-440
Values expressed as μg/g FW; UD, undetectable; FW, fresh weight

Plant number	Lutein	Zeaxanthin	β-carotene
DH12075	0.76	UD	0.30
DE19	1.48	UD	1.84
DE24	0.91	UD	0.25
DE25	0.17	UD	0.18
DE26	10.67	0.62	19.75
DE32	0.96	UD	2.08
DE35	2.68	UD	2.26
DE74	2.50	UD	1.52
DE89	7.82	0.36	13.07
DE91	0.75	UD	0.19
DE92	0.74	UD	0.19

Table 5. Concentration of carotenoids in seeds of transgenic B. napus plants expressing AtPSY
Values expressed as µg/g FW; UD, undetectable; FW, fresh weight

Plant line	β-Carotene	Lutein	Violaxanthin	Zeaxanthin	β-Cryptoxanthin	Total carotenoid	Ratio of β-carotene to lutein
DH12075	0.49±0.28	3.30±0.49	UD	UD	UD	5.34±0.96	0.15
construct 710-422							
BY173	15.07±4.98	39.10±5.33	2.43±0.18	0.44±0.13	0.23±0.04	75.48±11.23	0.39
BY228	19.52±3.27	41.74±3.80	1.30±0.11	2.51±0.69	0.08±0.13	86.59±8.57	0.47
BY241	4.18±0.28	14.05±0.21	0.61±0.02	0.21±0.02	0.10±0.07	24.31±0.56	0.30
BY269	90.76±6.72	76.22±3.74	1.74±0.07	7.07±0.13	0.30±0.13	227.78±2.85	1.19
BY351	6.36±4.14	17.44±3.46	1.09±0.32	0.14±0.03	UD	34.44±11.22	0.36
construct 710-423							
BY54	11.09±2.06	23.15±6.64	0.91±0.24	0.35±0.03	0.16±0.06	46.95±8.00	0.48
BY58	3.34±1.67	9.46±3.00	0.36±0.13	0.33±0.22	UD	19.07±5.82	0.35
BY223	17.88±1.25	25.29±1.16	1.48±0.12	0.31±0.05	0.15±0.01	59.81±3.19	0.71
BY365	27.02±4.04	37.64±3.95	2.42±0.25	1.73±0.33	0.15±0.03	94.09±12.07	0.72
BY371	3.50±0.31	10.36±0.57	0.45±0.07	0.28±0.01	UD	19.15±2.48	0.34

Table 6: Concentrations of carotenoid compounds in seeds of *B. napus* expressing ε-CYC RNAi and the untransformed control DH12075 line.

*Values expressed as µg/g FW; UD, undetectable; FW, fresh weight;
Each value is the mean result from triplicate±SD.

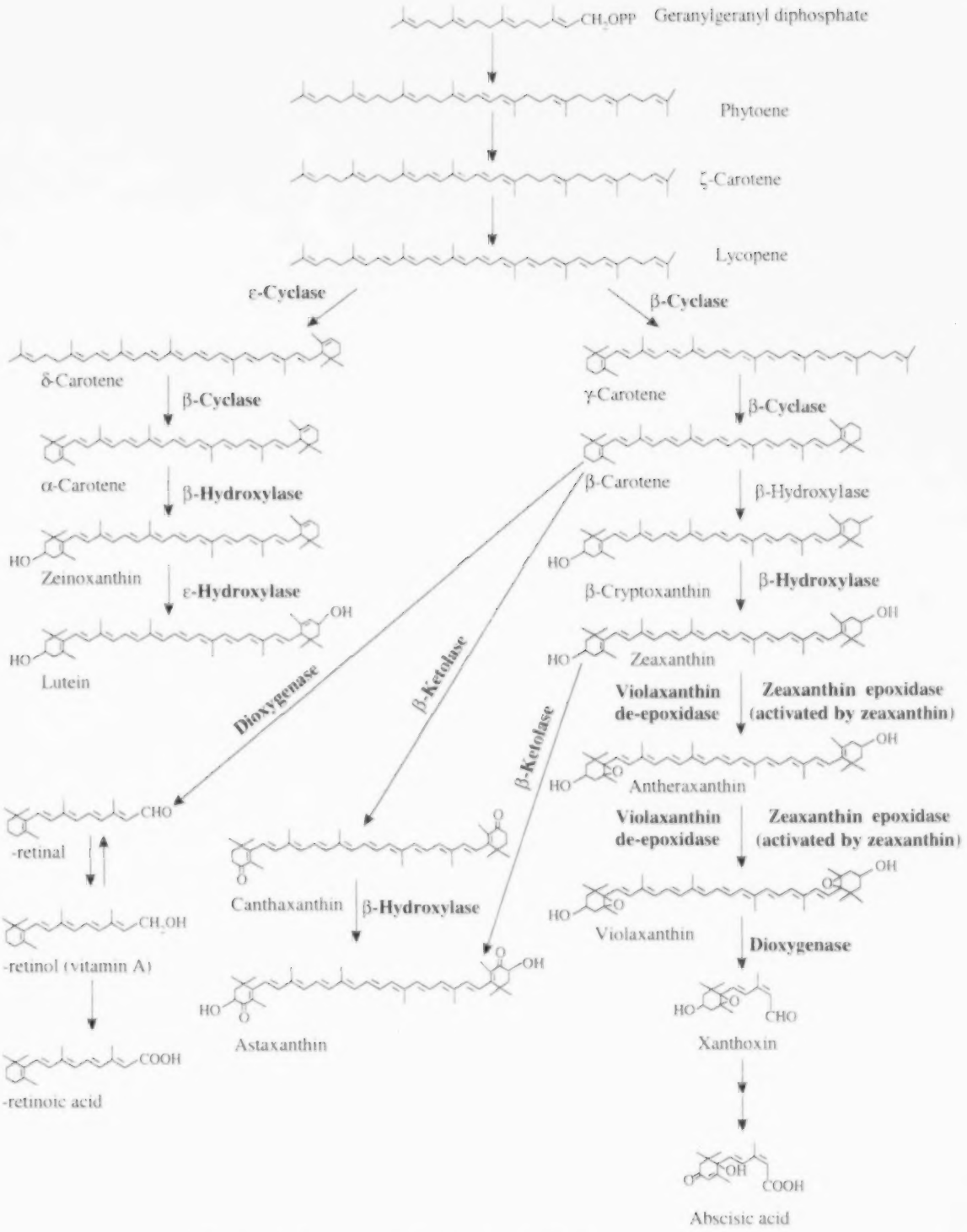


Figure 1: Carotenoid Biosynthesis Pathway

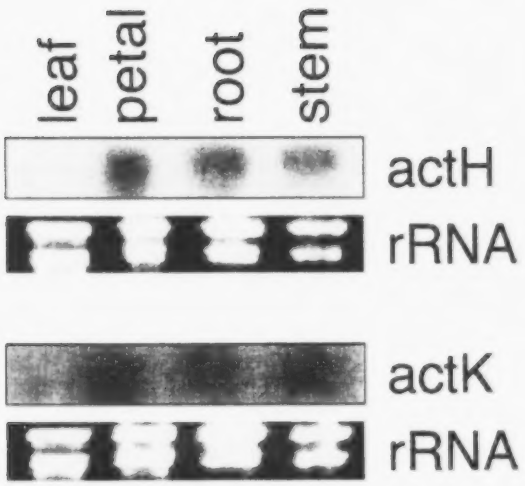


Figure 2: expression patterns of β-carotene hydroxylase and β-carotene ketolase in *A. aestivalis*.

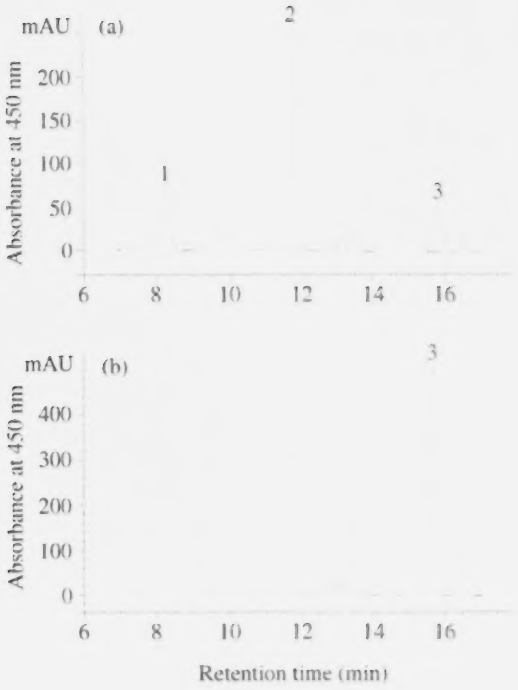
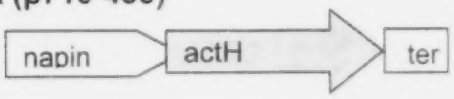


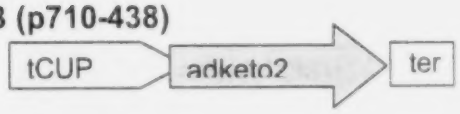
Figure 3 HPLC profiles of carotenoids extracted from *E. coli* cells carrying plasmids: (a) pBS-CrtH1 and pACCAR16ΔcrtX, (b) empty cloning vector pBluescriptII KS (+) and pACCAR16ΔcrtX. Peaks numbered 1 2 and 3 correspond to zeaxanthin, β-cryptoxanthin and β-carotene, respectively.

Figure 4: modified β-carotene hydroxylase (actH) and β-carotene ketolase (adket02) genes for expression in *B. napus*. A) β-carotene hydroxylase under control of the seed-specific napin promoter. B) β-carotene ketolase under control of the constitutive tCUP promoter. C) Both genes are in one construct.

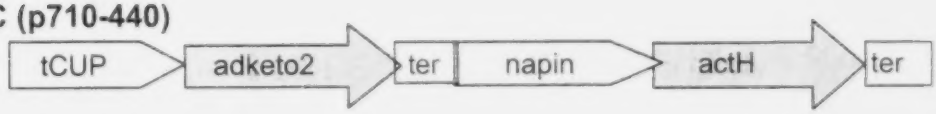
A (p710-433)



B (p710-438)



C (p710-440)



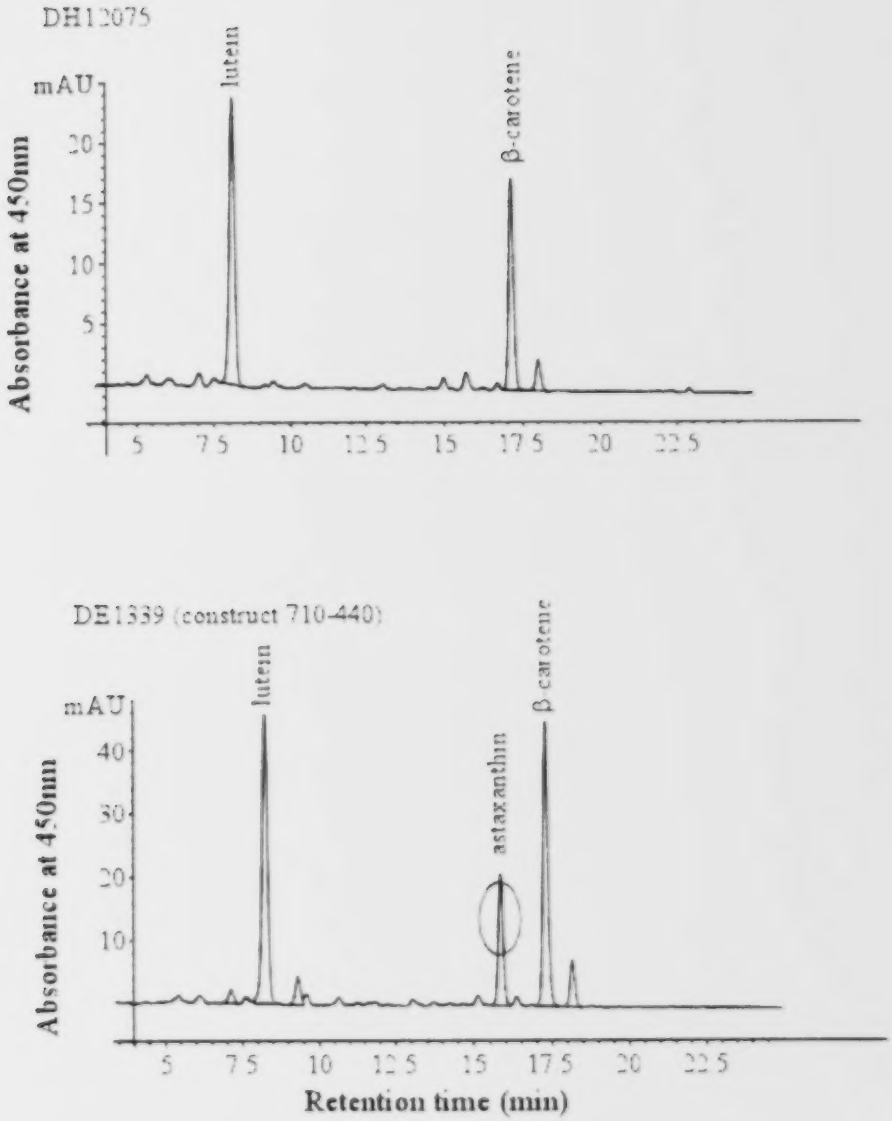


Figure 5: HPLC profiles of carotenoid extracts from seeds of B. napus DH12075 parental line, and line DE1339 expressing p710-440 construct harboring both actH and adKeto2. Astaxanthin peak is circled in red.

WT DE26 DE89

Figure 6: Visual comparison of seed carotenoid extracts from parental type (DH12075) and transgenic plants expressing *Arabidopsis* PSY gerne (DE26 and DE 89). The yellow colour in the extract from DE26 and DE89 is the result of increase in carotenoid concentration.

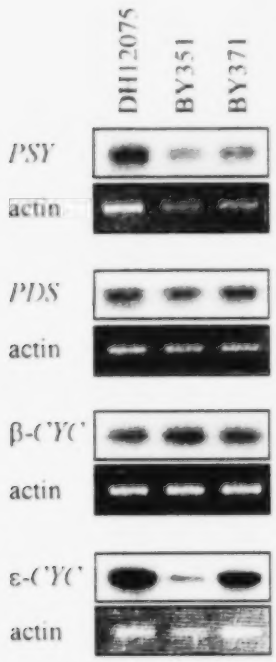


Figure 7: Carotenoid gene expression in developing seeds of DH12075 and *ε-CYC* RNAi lines, BY351 and 371. *PSY*, phytoene synthase; *PDS*, phytoene desaturase; β-*CYC*, lycopene β-cyclase; ε-*CYC*, lycopene ε-cyclase.

Figure 8. Visual comparison of carotenoid extracts from parental type (DH12075) and transgenic plants with RNAi-silenced ε-CYC line BY365.

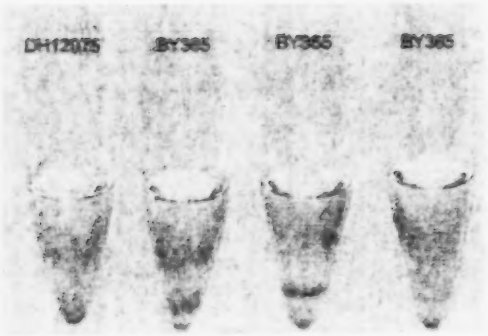


Figure 8. Visual comparison of carotenoid extracts from parental type (DH12075) and transgenic plants with RNAi-silenced ε-CYC line BY365.

Planta (2007) 226:181–192
DOI 10.1007/s00425-006-0455-1

ORIGINAL ARTICLE

Characterization of a β-carotene hydroxylase of *Adonis aestivalis* and its expression in *Arabidopsis thaliana*

Bianyun Yu · Derek J. Lydiate · Ulrike A. Schäfer · Abdelali Hannoufa

Received: 26 July 2006 / Accepted: 10 November 2006 / Published online: 14 December 2006
© Springer-Verlag 2006

Abstract Carotenoids are plant secondary metabolites that comprise two main groups: carotenes and xanthophylls. The latter group includes zeaxanthin which is synthesized by β-carotene hydroxylase catalyzing the hydroxylation of the β-rings of β-carotene molecules. To develop tools to alter carotenoid biosynthesis in plants, we isolated a cDNA clone encoding a candidate β-carotene hydroxylase, *CrtH1*, from the flower petals of *Adonis aestivalis*. CrtH1 protein has homology to β-carotene hydroxylases from other organisms, and possesses the four histidine motifs conserved in this family of enzymes. Sequence analysis predicted the presence of a putative plastid transit peptide at the amino terminus and four transmembrane helical regions. Southern-blot analysis showed *CrtH1* to be encoded by a multicopy gene family with at least three members in *A. aestivalis*. Analysis of *CrtH1* transcript abundance by Northern blotting indicates it is highly expressed in flower petals, roots and stems, with relatively low expression in leaves and developing seeds. CrtH1 was able to catalyze the formation of zeaxanthin and its intermediate precursor β-cryptoxanthin from β-carotene in functional assays conducted in *E. coli*. Expression of *CrtH1* in *Arabidopsis thaliana* wild type and a mutant deficient for endogenous β-carotene hydroxylases enhanced the biosynthesis of violaxanthin in the seeds.

The sequence of the β-carotene hydroxylase, CrtH1, has been submitted to Gene Bank, accession #EF120636.

B. Yu · D. J. Lydiate · U. A. Schäfer · A. Hannoufa (✉)
Agriculture and Agri-Food Canada,
Saskatoon Research Centre, 107 Science Place,
Saskatoon, SK, S7N 0X2Canada
e-mail: hannoufaa@agr.gc.ca

Keywords *Adonis aestivalis* · β-Carotene hydroxylase · Carotenoids · Xanthophylls

Abbreviations

BHT	Butylated hydroxytoluene
DEPC	Diethylpyrocarbonate
IPTG	Isopropyl-β-D-thiogalactopyranoside
ORF	Open-reading frame
TAG	Triacylglyceride

Introduction

Carotenoids are ubiquitous secondary metabolites and pigments responsible for some organ coloration in plants and microorganisms (Ausich 1997). Carotenoids are used as colorants in the food industry (Fraser 1997) and as essential supplements in livestock and fish feed formulations (Johnson and Schroeder 1996). The importance of carotenoids in human health is well documented and includes roles as antioxidants with potential anti-cancer therapeutic properties (Mayne 1996).

Carotenoids are synthesized in all photosynthetic plants, algae and cyanobacteria, and act as important components of the photosynthetic machinery, where they act as photoprotecting antioxidants and regulate membrane fluidity (Umeno et al. 2005). Many non-photosynthetic bacteria (*Erwinia herbicola, Thermus aquaticus*) and fungi (*Neurospora crassa*) also synthesize carotenoids. In plants, carotenoids are synthesized and localized together with chlorophyll in chloroplasts, or alone in the chromoplasts of flowers, fruits and other tissues (Giuliano et al. 2000; Römer et al. 2002). Based on their structural features, carotenoids are divided into two major groups: carotenes and

xanthophylls. Carotenes are hydrocarbons that are either linear, like lycopene, or cyclized, like α-carotene and β-carotene. Xanthophylls are oxygenated carotenes, which can contain hydroxyl, epoxy or keto groups, and include lutein, zeaxanthin, violaxanthin and astaxanthin (Tian and DellaPenna 2001). Xanthophylls comprise most of the carotenoid pigment in the photosynthetic tissues of plants (Cunningham and Gantt 1998) and contribute to the assembly and stability of pigment-protein complexes, light-harvesting and photoprotection in chloroplasts (Hirschberg 2001; Rissler and Pogson 2001). Xanthophylls are also precursors for the biosynthesis of the phytohormone abscisic acid (ABA) (Giuliano et al. 2000).

Carotenoids are derived from the isoprenoid pathway in which the condensation of five-carbon isoprenoid units to form phytoene represents the first committed step in carotenoid biosynthesis (Taylor and Ramsay 2005). This is followed by the desaturation of phytoene to lycopene and two cyclization reactions converting lycopene to either α- or β-carotene (Cunningham and Gantt 1998). Hydroxylation of cyclic carotenes at the 3C, 3′C positions can be carried out by two types of hydroxylases: β- and ε-hydroxylase, which add a hydroxyl group to β- and ε-ring, respectively. Consequently, β-carotene is converted to zeaxanthin and α-carotene to lutein (Fig. 1; Giuliano et al. 2000; Hirschberg 2001; Tian and DellaPenna 2004). Lutein (3R, 3R′-β, ε-carotene-3, 3′-diol) is the most abundant carotenoid in all plant photosynthetic tissues and protects the photosynthetic system from damage by strong light. Zeaxanthin (3R, 3R′-β, β-carotene-3, 3′-diol) plays an important role in non-photochemical quenching, which helps to protect plants against damage from singlet oxygen and other reactive oxygen (Rissler and Pogson 2001). Zeaxanthin also acts as a lipid stabilizer and protects against lipid peroxidation (Rissler and Pogson 2001; Müller-Moulé et al. 2003). Transgenic *Arabidopsis thaliana* plants overexpressing β-carotene hydroxylase are more tolerant to conditions of high light and high temperature (Davison et al. 2002).

The genes encoding β-carotene hydroxylases have been cloned from bacteria, green algae and higher plants (Hundle et al. 1993; Sun et al. 1996; Bouvier et al. 1998; Linden 1999; Kim et al. 2001; Tian and DellaPenna 2001). Two β-carotene hydroxylases have been identified in *A. thaliana* (Sun et al. 1996; Tian and DellaPenna 2001) and both acted effectively on the β-ring but poorly on the ε-ring. Sun et al. (1996) suggested that β-carotene hydroxylase of *A. thaliana* normally associates with a second β-carotene hydroxylase, or with an ε-hydroxylase, to form a dimer, and a portion of the N-terminus mediates these interactions. Bouvier et al. (1998) isolated two β-carotene hydroxylases from pepper fruits. Both enzymes were shown to be ferredoxin dependent proteins, and the histidine motifs conserved among this group of enzymes were suggested to be the iron ligands essential for activity. In-vitro reconstitution experiments indicated that pepper β-carotene hydroxylase used iron-activated oxygen to break the C–H bond with the formation of a double bond or oxygen insertion (Bouvier et al. 1998).

Flowers of *Adonis aestivalis* are known to accumulate high levels of xanthophylls (Seybold and Goodwin 1959)

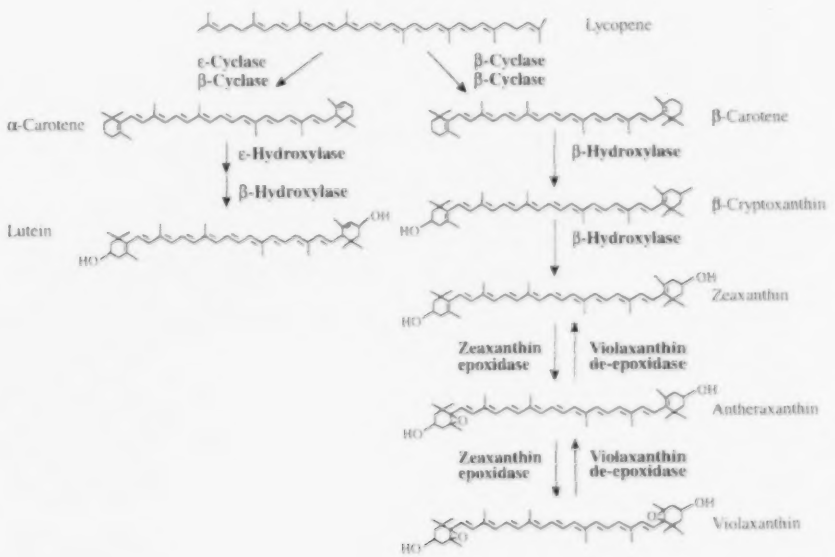


Fig. 1 Carotenoid biosynthesis pathway in plants

and are therefore an ideal source of genes involved in synthesizing and modifying these metabolites. However, no β-carotene hydroxylases have so far been cloned from this plant species. In an effort to develop tools and strategies for altering the carotenoid profiles of plants, we cloned a cDNA, *CrtH1*, from the flower petals of *A. aestivalis*. The *CrtH1* cDNA encodes a predicted protein with homology to published β-carotene hydroxylases that catalyze the hydroxylation of the β-rings of β-carotene to give rise to zeaxanthin. The biochemical function of CrtH1 was determined by functional assays in a β-carotene-producing *E. coli* strain. The carotenoid profile of *A. thaliana* seeds was significantly altered upon the expression of *CrtH1* in *A. thailana* wild type and a mutant deficient in both endogenous β-carotene hydroxylases (Tian et al. 2003). These results are discussed in relation to the possible use of *CrtH1* to engineer carotenoid metabolism in plants.

Materials and methods

Plant materials

Seeds of *A. aestivalis* L. were purchased from Thompson & Morgan Ltd. (Ipswich, Suffolk, UK). *A. aestivalis* plants were grown in RediEarth (W.R. Grace & Co., Ajax, Canada) soil in a controlled greenhouse environment (16 h light/8 h dark, 20/17°C).

Wild type *Arabidopsis thaliana* (ecotype Wassilewskija) and homozygous *A. thaliana* b1b2 mutant with T-DNA knockouts in two β-carotene hydroxylase genes (Tian et al. 2003) were grown in Co–Co soil-less mixture under controlled greenhouse environment (16 h light/8 h dark, 20°/17°C). Seeds of *A. thaliana* b1b2 mutant were kindly provided by Dr. Dean DellaPenna (Michigan State University, East Lansing, MI, USA). *A. thaliana* transformation was carried out according to the floral dip procedure described by Clough and Bent (1998). Transgenic plants were selected by spraying with the herbicide glufosinate at 150 g/l (Liberty), and the presence of the transgene was confirmed by PCR using forward primer, 5′-GCTTTC TTCATCGGTGATTGATTCC-3′ and reverse primer, 5′-GCGAGCTCTAAGGCATTCATACGCTTTAT TCTTC-3′.

Chemical standards

β-carotene and lutein were purchased from Sigma (Oakville, Canada). β-cryptoxanthin, zeaxanthin and violaxanthin were purchased from CaroteNature (Lupsingen, Switzerland).

Cloning of *CrtH1* cDNA of *Adonis aestivalis*

Degenerate primers for amplifying *CrtH1* were designed based on the conserved domains of β-carotene hydroxylases of various organisms: forward primer, 5′-GTTGG(C/T)GCTGC(C/T)GT(A/T)GG (A/T/G)ATGGA-3′; and reverse primer, 5′-(C/T)TT (A/G)TC(A/T)GTGTGGTG(A/T)AGCTGGTG(A/ G)G-3′. These primers were used to amplify a fragment of *CrtH1* by PCR using as template cDNA synthesized from RNA isolated from the flower petals of *A. aestivalis* according to the manufacture's instruction for reverse transcriptase (Invitrogen, Burlington, Canada). A 363 bp PCR product was blunt-end cloned into the *Eco*RV site of pBluescipt KS (+) vector, and sequenced. The 5′- and 3′-ends of the *CrtH1* cDNA were isolated by employing 5′- and 3′-rapid amplification of cDNA ends (RACE) techniques according to the manufacturers' instructions (5′-RACE system for rapid amplification of cDNA Ends, Version 2.0, Invitrogen; and 3′-RACE, First Choice RLM-RACE, Ambion, Austin, TX, USA).

Sequence analysis of *CrtH1*

Alignment of the predicted amino acid sequence encoded by the *CrtH1* cDNA and its phylogenic relationship with β-carotene hydroxylases from various organisms was conducted using Vector NTI program (InforMax, Invitrogen). The chloroplast transit peptide was predicted using the online software Chloroplast v1.1 (http://www.cbs.dtu.dk/services/ChloroP/). Transmembrane helices were predicted using TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

DNA isolation and Southern-blot analysis

Total genomic DNA was isolated from leaves of *A. aestivalis* using DNeasy Plant Mini Kit (Qiagen, Mississauga, Canada). Approximately 10 μg of genomic DNA was digested with each of *Bam*HI, *Hind*III, *Kpn*I, *Spe*I and *Sst*I restriction endonucleases, separated on a 0.8% agarose gel and transferred to Hybond-XL membrane (Amersham Biosciences, Quebec, Canada). The blot was probed with a 201 bp ^{32}P-labeled *CrtH1*-specific fragment obtained by PCR using the forward primer 5′-AGAAGGAGATAG AGCGAAGAATAAAGCGT-3′, and the reverse primer 5′-ACATGTACACGTTGTGAGTACGAC TCTT-3′. Hybridization was performed with Church buffer (Church and Gilbert 1984) at 61°C for 22 h. The filter was washed twice in 2× SSC, 0.1% SDS for 10 min at 61°C and followed by washing twice in

0.2× SSC, 0.1% SDS for 10 min at 61°C. The filter was then exposed to an X-ray film with an intensifying screen at −70°C for 7 days.

RNA isolation and Northern-blot analysis

Total RNA was isolated from *A. aestivalis* leaves, flower petals, roots, stems and developing seeds as described by Carpenter and Simon (1998) with some modifications. After grinding in liquid nitrogen, about 100 mg of tissue was extracted with 600 ml of RNA extraction buffer (0.2 M Tris–HCl, pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% SDS) and an equal volume of Tris–HCl buffered phenol (pH 7.9). Extraction was repeated twice with phenol and followed once with chloroform. Approximately 1/4 volume of 10 M LiCl was added to the aqueous layer, mixed well, stored at 4°C overnight and then centrifuged at 14,000*g* for 20 min. The pellet was resuspended in 0.3 ml of DEPC-treated dH_2O followed by 30 µl of 3 M sodium acetate (pH 5.3) and 0.7 ml of 95% ethanol. The mixture was chilled at −70°C for 10 min and then centrifuged at 14,000 *g* for 20 min. The pellet was washed with 75% ethanol and re-suspended in 20 µl of DEPC-treated dH_2O. A total of 15 µg of total RNA was electrophoresed on 1.2% agarose gel containing formaldehyde and transferred onto Hybond-XL membrane (Amersham Biosciences). The hybridization method and probe used were as described for Southern-blot analysis.

Vector construction for functional assays in *E. coli* and *A. thaliana*

Plasmid pACCAR16ΔcrtX containing *Erwinia uredovora* genes required to produce β-carotene was constructed by Misawa et al. (1995) and was kindly provided to us by Dr. Hartmut Linden (Universitaet Konstanz, Germany). This plasmid was introduced into *E. coli* JM101 strain by electroporation. The open reading frame (ORF) of *CrtH1* was amplified by PCR using the forward primer, 5′-gc**ggatcc**aATGCTAGCT TCAATGGCAGCGGCA-3′, which incorporates a *Bam*HI site (in bold) and the reverse primer, 5′-gc**gag-ctc**CTATAAGGCATTCATACGCTTTATTCTTC-3′, which incorporates a *Sac*I site (in bold). The PCR product was directionally cloned between the *Bam*HI and *Sac*I sites of pBluescriptII KS (+) vector to produce plasmid pBS-CrtH1, and then introduced into the JM101 strain carrying plasmid pACCAR16ΔcrtX. The empty cloning vector pBluescriptII KS (+) was also introduced into the JM101 strain with plasmid pACCAR16ΔcrtX as a negative control.

For expression of *CrtH1* in *A. thaliana* under the control of the seed-specific napin promoter from *Brassica napus* (Rask et al. 1998), the 930 bp ORF fragment of *CrtH1* was amplified by PCR using the following forward and reverse primers having built-in *Bam*HI and *Sac*I sites, respectively: forward 5′-GCGGATCCAA TGCTAGCTTCAATGGCAGCGGCA-3′, and reverse 5′-GCGAGCTCCTATAAGGCATTCATACGCTT TATTCTTC-3′. The PCR product was digested with *Bam*HI and *Sac*I and ligated between the *Bam*HI and *Sac*I sites of pBluescriptII KS (+) vector, in which the napin promoter of *Brassica napus* was cloned between the *Hind*III and *Bam*HI sites. A ~2.1 kb fusion fragment of the napin promoter and the ORF of *CrtH1* was then excised by digestion with *Hind*III and *Sac*I, and cloned between the *Hind*III and *Sac*I sites of an in house-built vector, p79–103, harbouring a *BAR* gene for glyphosinate selection in plants (Fig. 2).

Functional assay in *E. coli* and HPLC analysis

Assay of CrtH1 activity in *E. coli* and HPLC analysis were adapted from Misawa et al. (1995) and Shewmaker et al. (1999). Cultures of *E. coli* JM101 carrying different plasmids were grown in LB broth with antibiotics (34 µg/ml chloramphenicol and 150 µg/ml ampicillin) in the dark at 28°C overnight. For each *E. coli* strain, 2 ml of the overnight culture was used to inoculate 75 ml of LB broth media containing antibiotics. These cultures were grown in the dark at 28°C for 3 h, and then IPTG was added to a final concentration of 0.5 mM before further incubation for 24 h. Each culture was aliquoted into triplicates of 25 ml each, then

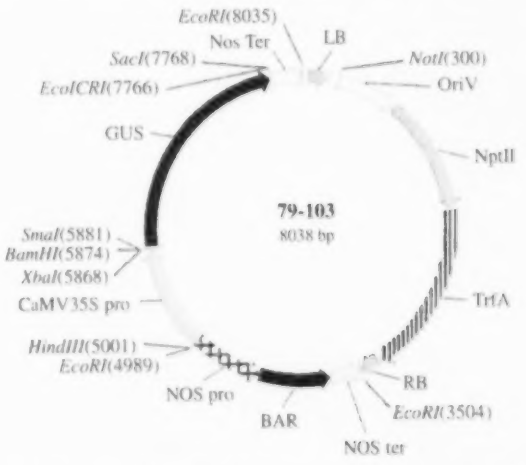


Fig. 2 Diagram of the p79–103 plant transformation vector. The BAR gene for glyphosinate selection in plants is driven by the NOS promoter

cells were harvested by centrifugation and the pellets were either immediately subjected to carotenoid extraction or were frozen at −80°C. Carotenoid extraction was carried out by adding 10 ml acetone to the pellet and mixing well by vortexing. The mixture was incubated at 55°C for 10 min and centrifuged at 11,000*g* for 10 min. The supernatant was transferred to a clean tube and dried down at room temperature under a stream of N_2 gas. The residue was re-suspended in 5 ml methanolic-KOH (10 g KOH per 100 ml in methanol/water, 80/20, v/v) and incubated at room temperature for 15 min to saponify the triacylglycerides (TAG) in the extract so they partition to the aqueous phase. A total of 2 ml H_2O and 3 ml petroleum ether were then added to extract carotenoids. Ether extraction was repeated once, and the extracts were pooled and dried down at room temperature under a N_2 gas stream. The residue was resuspended in 200 µl of acetonitrile/methylene chloride/methanol (50/40/10, by vol) with BHT (butylated hydroxytoluene, 0.5% (w/v) and filtered through a 0.2 µm pore size 4 mm nylon syringe filter into an HPLC sample vial. The extract was immediately subjected to analysis by HPLC-PDA. Aliquots of 20 µl were analyzed by HPLC employing a YMC "Carotenoid Column"—a reverse-phase C_{30}, 5 µm column (4.6 × 250 mm; Waters Ltd, Mississauga, Canada) with a column temperature of 35°C. Mobile phases consisted of methanol (A) and *tert*-methyl butyl ether (B). The gradient elution used with this column started at 95% A and 5% B, and then followed by a linear gradient to 35% A and 65% B in 25 min. A flow rate of 1.2 ml/min was used, and the eluate was monitored at 450 nm. Peaks were identified by their retention time and absorption spectra compared to those of known standards. Quantification of carotenoids was conducted using curves constructed from authentic standards.

Extraction of carotenoids from *A. thaliana* seeds and HPLC analysis

Approximately 200 mg of seed (weighed accurately to four decimal places) was combined with 3 ml of hexane/acetone/ethanol (50:25:25, by vol) extraction solvent in a scintillation vial (adapted from Shewmaker et al. 1999). A steel rod was put in the vial and the sample was pulverized for 30 min with rapid shaking. The sample was then centrifuged for 10 min at 1,800*g*, and the supernatant was transferred to a clean Kimax screw-cap culture tube. Another 3 ml of extraction solvent was added to the pellet and centrifugation was repeated. The extracts were pooled and dried down at room temperature under a stream of N_2 gas. The residue was re-suspended in 5 ml methanolic-KOH (10 g KOH per 100 ml in methanol/water, 80/20, v/v) and heated at 80°C for 1 h. Subsequent steps were the same as those described above for the extraction and analysis of carotenoids in the *E. coli* functional assay.

Results and discussion

CrtH1 encodes β-carotene hydroxylase in *Adonis aestivalis*

Alignment of the amino acid sequences of β-carotene hydroxylases of various organisms revealed two highly conserved amino acid motifs: VGAAVGME and AHQLHHTDK (Fig. 3). A pair of degenerate primers corresponding to the two conserved sequences was used to amplify a 363 bp fragment by RT-PCR using RNA extracted from the flower petals of *A. aestivalis*. The 5′- and 3′-ends of cDNA were then isolated using respective RACE techniques. The assembly of the three fragments produced a cDNA clone of 1,187 bp representing *CrtH1*. The cDNA encoded a predicted protein of 309 amino acids with a molecular weight of ~35 kDa and a pI of 9.15.

An alignment of the deduced protein sequence of CrtH1 with those of β-carotene hydroxylases from other plants showed that they shared high level of similarity, including ~70% amino acid identity, with most of the diverging sequence residing in the amino terminus (Fig. 4). However, CrtH1 showed much lower homology with β-carotene hydroxylases from bacteria (~33% identity; data not shown). Phylogenic analysis indicates that CrtH1 is most similar to the β-carotene hydroxylases from *Citrus unshiu*, and further highlights the divergence between the plant and prokaryotic enzymes (Fig. 5). The chloroplast transit peptide prediction software Chloroplast v1.1 (http://www.cbs.dtu.dk/services/ChloroP/) predicted a chloroplast transit peptide cleavage site at the amino terminus of CrtH1 between Val^{59} and Ala^{60}. This is consistent with the presumed plastid location of a carotenoid biosynthetic enzyme since carotenoid biosynthesis and modification in plants take place in plastids (Cunningham and Gantt 1998). Both β-carotene hydroxylase−1 and -2 of *A. thaliana* have chloroplast transit peptides in their N-terminus (Tian and DellaPenna 2001). The N-terminal region of about 100 amino acids in plant β-carotene hydroxylases is highly divergent, and is much longer than typical chloroplast transit peptide signals (Sun et al. 1996). It has been suggested that a portion of the amino terminus of the β-carotene hydroxylases of *A. thaliana* may be involved in mediating the formation

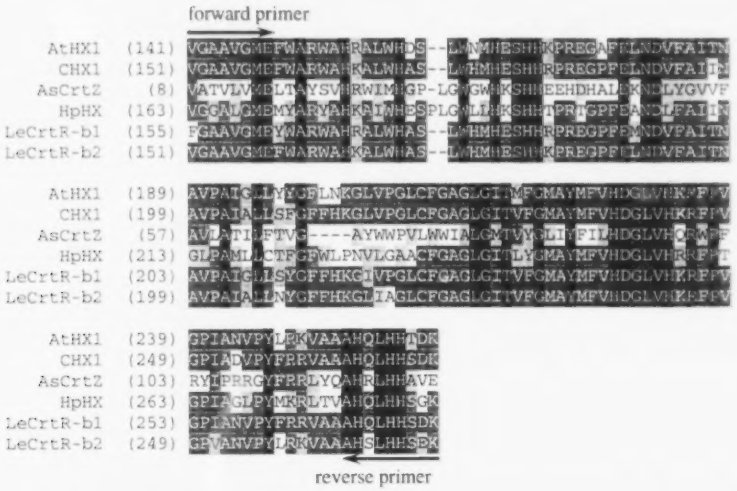


Fig. 3 Alignment of amino acid sequences of β-carotene hydroxylases of various organisms, and positions of the degenerate primers used to amplify the conserved 363 bp fragment. Identical and highly conserved amino acids in the six sequences are shown as *white letters* on *black* and *gray* backgrounds, and amino acids with similarity are indicated as *black letters* on a *gray* background. Amino acids with no similarity are shown as *black letters* on a *white background*. GenBank accession numbers of these sequences are as follows: *Lycopersicon esculentum LeCrtR*-b1 (Y14809) and *LeCrtR*-b2 (Y14810); *Alcaligenes sp. AsCrtZ* (D58422); *Arabidopsis thaliana AtHX*1 (AF370220); *Citrus unshiu CHX*1(AF296158); *Haematococcus pluvialis HpHX* (AF162276)

of dimers of β-carotene hydroxylase molecules (Sun et al. 1996), which is likely a common feature of plant hydroxylases.

Four transmembrane helical regions were predicted by TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) in CrtH1 (Fig. 4). Four conservatively spaced histidine motifs were also present, HXXXXH and HXXH (Fig. 4). The histidine motifs are characteristic of membrane fatty acid desaturases and membrane hydrocarbon hydroxylases, where they are proposed to be involved in iron binding during hydroxylation reactions; mutations in any one of the ten histidine residues resulted in complete loss of the enzyme activity of β-carotene hydroxylase from pepper fruits (Bouvier et al. 1998). It is important to note that CrtH1 shares significant homology with the two β-carotene ketolases of *A. aestivalis*, AdKeto1 and AdKeto2, with ~70% amino acid identity (data not shown). However, neither AdKeto1 nor AdKeto2 showed either 4-ketolase or 3-hydroxylase activity when β-carotene was used as a substrate in *E. coli* functional assays. Both enzymes modified β-rings to form 4-keto-β-ring via an indirect route; keto-enol tautomerization (Cunningham and Gantt 2005).

CrtH1 is encoded by a multi-gene family in *Adonis aestivalis*

To estimate the *CrtH1* gene copy number in the *A. aestivalis* genome, we carried out Southern-blot analysis on total genomic DNA digested with restriction endonucleases using a 201 bp *CrtH1*-specific fragment as a probe under high stringency conditions (Fig. 6). Digestion with *Bam*HI, *Hin*dIII, *Kpn*I, *Spe*I *and Sst*I, none of which cleaves within the ORF of *CrtH1*, resulted in the detection of at least three bands. The results suggest that *CrtH1* belongs to a gene family of at least three members in the *A. aestivalis* genome (Fig. 6). This is similar to findings in other plants. In *A. thaliana*, β-carotene hydroxylase is encoded by two genes (Sun et al. 1996; Tian and DellaPenna 2001). The β-carotene hydroxylases, b1 and b2, of *A. thaliana* are functionally redundant, but with specialized functions (Tian et al. 2003). As well, two β-carotene hydroxylases, *Ca1* and *Ca2*, have been isolated from pepper fruits (Bouvier et al. 1998), citrus (Kim et al. 2001) and tomato (Hirschberg 1998). This suggests that the existence of multi-copy gene families of β-carotene hydoxylases is likely of common occurrence in plants.

CrtH1 is differentially expressed in *A. aestivalis* organs

Northern-blot analysis was carried out to investigate the expression profile of the *CrtH1* gene in different organs of *A. aestivalis*. The *CrtH1* transcript (~1.35 kb) was highly abundant in flower petals, roots and stems with relatively much lower abundance in leaves and developing seeds (Fig. 7). This expression pattern is consistent with reports in the literature on

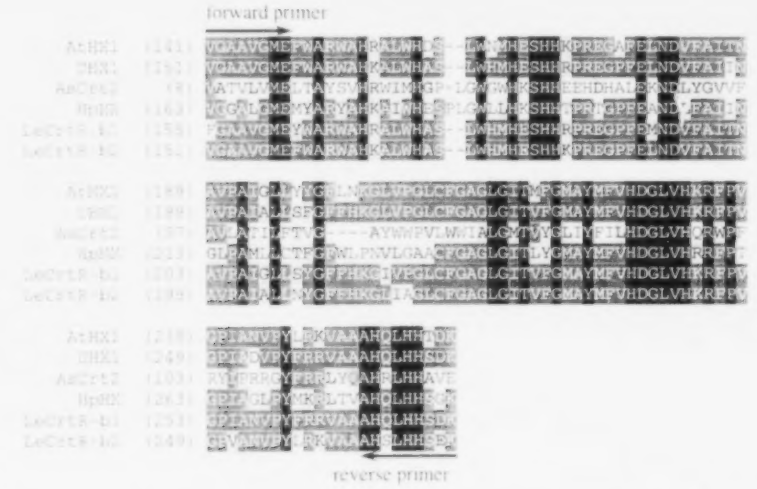


Fig. 3 Alignment of amino acid sequences of β-carotene hydroxylases of various organisms, and positions of the degenerate primers used to amplify the conserved 363 bp fragment. Identical and highly conserved amino acids in the six sequences are shown as *white letters* on *black* and *gray* backgrounds, and amino acids with similarity are indicated as *black letters* on a *gray* background. Amino acids with no similarity are shown as *black letters* on a *white background*. GenBank accession numbers of these sequences are as follows: *Lycopersicon esculentum LeCrtR*-b1 (Y14809) and *LeCrtR*-b2 (Y14810); *Alcaligenes sp. AsCrtZ* (D58422); *Arabidopsis thaliana AtHX1* (AF370220); *Citrus unshiu CHX1* (AF296158); *Haematococcus pluvialis HpHX* (AF162276)

of dimers of β-carotene hydroxylase molecules (Sun et al. 1996), which is likely a common feature of plant hydroxylases.

Four transmembrane helical regions were predicted by TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) in CrtH1 (Fig. 4). Four conservatively spaced histidine motifs were also present, HXXXXH and HXXH (Fig. 4). The histidine motifs are characteristic of membrane fatty acid desaturases and membrane hydrocarbon hydroxylases, where they are proposed to be involved in iron binding during hydroxylation reactions; mutations in any one of the ten histidine residues resulted in complete loss of the enzyme activity of β-carotene hydroxylase from pepper fruits (Bouvier et al. 1998). It is important to note that CrtH1 shares significant homology with the two β-carotene ketolases of *A. aestivalis*, AdKeto1 and AdKeto2, with ~70% amino acid identity (data not shown). However, neither AdKeto1 nor AdKeto2 showed either 4-ketolase or 3-hydroxylase activity when β-carotene was used as a substrate in *E. coli* functional assays. Both enzymes modified β-rings to form 4-keto-β-ring via an indirect route: keto-enol tautomerization (Cunningham and Gantt 2005).

CrtH1 is encoded by a multi-gene family in *Adonis aestivalis*

To estimate the *CrtH1* gene copy number in the *A. aestivalis* genome, we carried out Southern-blot analysis on total genomic DNA digested with restriction endonucleases using a 201 bp *CrtH1*-specific fragment as a probe under high stringency conditions (Fig. 6). Digestion with *Bam*HI, *Hin*dIII, *Kpn*I, *Spe*I *and Sst*I, none of which cleaves within the ORF of *CrtH1*, resulted in the detection of at least three bands. The results suggest that *CrtH1* belongs to a gene family of at least three members in the *A. aestivalis* genome (Fig. 6). This is similar to findings in other plants. In *A. thaliana*, β-carotene hydroxylase is encoded by two genes (Sun et al. 1996; Tian and DellaPenna 2001). The β-carotene hydroxylases, b1 and b2, of *A. thaliana* are functionally redundant, but with specialized functions (Tian et al. 2003). As well, two β-carotene hydroxylases, *Ca1* and *Ca2*, have been isolated from pepper fruits (Bouvier et al. 1998), citrus (Kim et al. 2001) and tomato (Hirschberg 1998). This suggests that the existence of multi-copy gene families of β-carotene hydoxylases is likely of common occurrence in plants.

CrtH1 is differentially expressed in *A. aestivalis* organs

Northern-blot analysis was carried out to investigate the expression profile of the *CrtH1* gene in different organs of *A. aestivalis*. The *CrtH1* transcript (~1.35 kb) was highly abundant in flower petals, roots and stems with relatively much lower abundance in leaves and developing seeds (Fig. 7). This expression pattern is consistent with reports in the literature on

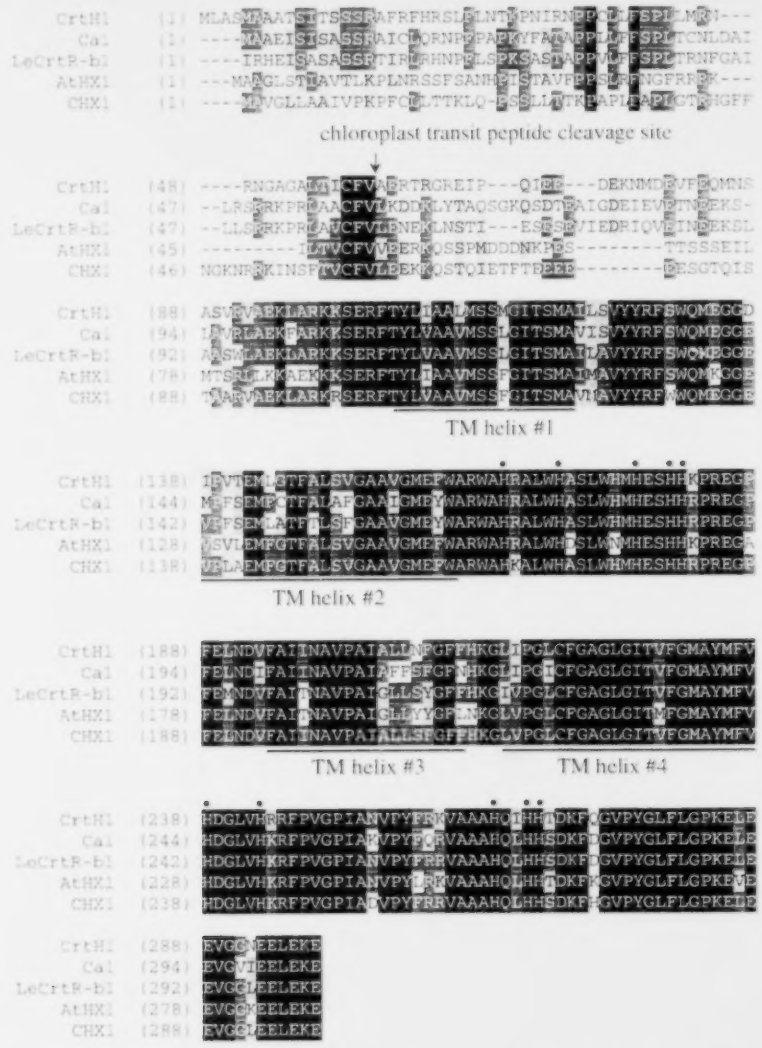


Fig. 4 Alignment of the deduced amino acid sequence of CrtH1 to those of β-carotene hydroxylases of various organisms. Shadings are as indicated in Fig. 3. GenBank accession numbers of these sequences are: *Arabidopsis thaliana AtHX1*(AF370220); *Capsicum annuum Ca1* (Y09225); *Citrus unshiu CHX1*(AF296158); *Lycopersicon esculentum LeCrtR*-b1 (Y14809). *TM* transmembrane helices; *shaded circle* conserved β-carotene hydroxylase histidine residues

other β-carotene hydroxylases. For example, experiments in *A. thaliana* using antisense suppression of β-carotene hydroxylase indicated that only a few molecules of the total zeaxanthin in wild type plants were required for optimal chlorophyll fluorescence quenching in vivo (Rissler and Pogson 2001). This suggests that low level of β-carotene hydroxylase expression is likely sufficient in plant leaves. During the development of flowers, plastids differentiate into chromoplasts that accumulate high levels of carotenoids. In the flower petals of tomato plants, the process of plastid differentiation to chromoplasts resulted in more than eightfold increase in carotenoid content (Giuliano et al. 1993), and in pepper fruits chloroplast to chromoplast transition stage corresponded with strong induction of the expression of β-carotene hydroxylase (Bouvier et al. 1998). During marigold petal development, considerably higher level of β-carotene hydroxylase transcript accumulated in the petal than in the leaf (Moehs et al. 2001). Yellow carotenoids (xanthophylls) are responsible for the colors of petals and anthers (Giuliano et al. 1993). This suggests that more β-carotene hydroxylase is required during petal development, which is consistent with the relatively higher level of expression of *CrtH1* in petals of *A. aestivalis*.

In addition to the *CrtH1* transcript of ~1.35 kb, two other transcripts having sizes of ~2.37 and ~0.900 kb were also detected in petals, roots and stems, but not in leaves and seeds. The detection of three transcripts is in accordance with the Southern blotting data (Fig. 6), in which three *CrtH1* homologues were predicted to be encoded by the *A. aestivalis* genome.

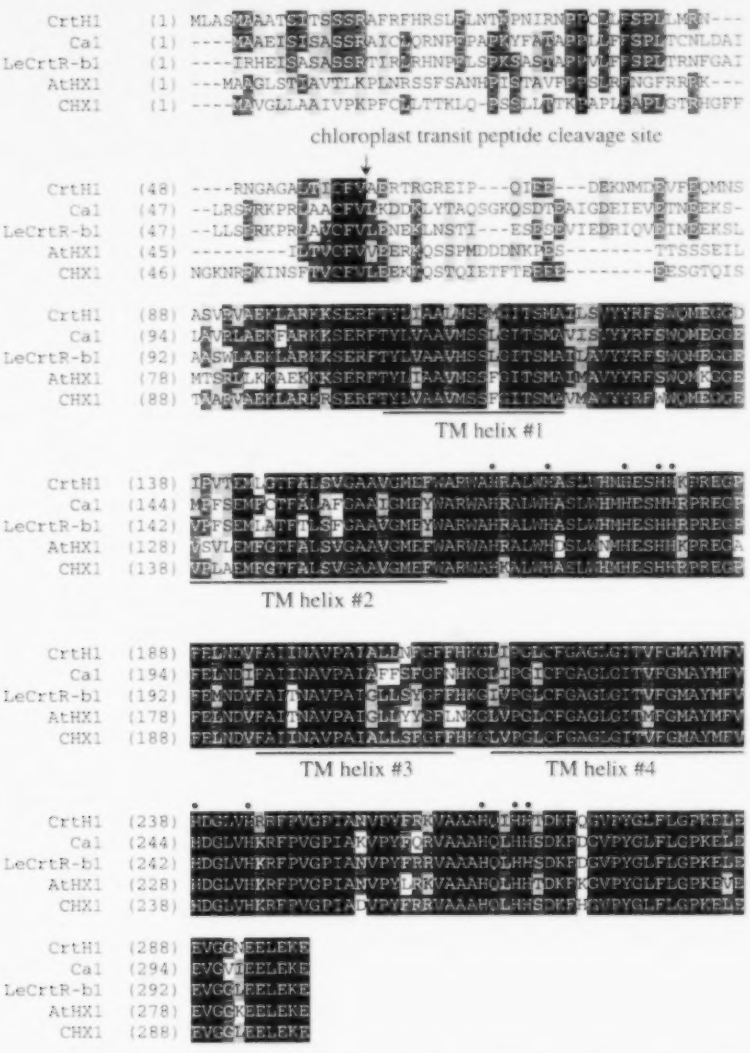


Fig. 4 Alignment of the deduced amino acid sequence of CrtH1 to those of β-carotene hydroxylases of various organisms. Shadings are as indicated in Fig. 3. GenBank accession numbers of these sequences are: *Arabidopsis thaliana AtHX1*(AF370220); *Capsicum annuum Ca1* (Y09225); *Citrus unshiu CHX1*(AF296158); *Lycopersicon esculentum LeCrtR*-b1 (Y14809). *TM* transmembrane helices; *shaded circle* conserved β-carotene hydroxylase histidine residues

other β-carotene hydroxylases. For example, experiments in *A. thaliana* using antisense suppression of β-carotene hydroxylase indicated that only a few molecules of the total zeaxanthin in wild type plants were required for optimal chlorophyll fluorescence quenching in vivo (Rissler and Pogson 2001). This suggests that low level of β-carotene hydroxylase expression is likely sufficient in plant leaves. During the development of flowers, plastids differentiate into chromoplasts that accumulate high levels of carotenoids. In the flower petals of tomato plants, the process of plastid differentiation to chromoplasts resulted in more than eightfold increase in carotenoid content (Giuliano et al. 1993), and in pepper fruits chloroplast to chromoplast transition stage corresponded with strong induction of the expression of β-carotene hydroxylase (Bouvier et al. 1998). During marigold petal development, considerably higher level of β-carotene hydroxylase transcript accumulated in the petal than in the leaf (Moehs et al. 2001). Yellow carotenoids (xanthophylls) are responsible for the colors of petals and anthers (Giuliano et al. 1993). This suggests that more β-carotene hydroxylase is required during petal development, which is consistent with the relatively higher level of expression of *CrtH*1 in petals of *A. aestivalis*.

In addition to the *CrtH*1 transcript of ~1.35 kb, two other transcripts having sizes of ~2.37 and ~0.900 kb were also detected in petals, roots and stems, but not in leaves and seeds. The detection of three transcripts is in accordance with the Southern blotting data (Fig. 6), in which three *CrtH*1 homologues were predicted to be encoded by the *A. aestivalis* genome.

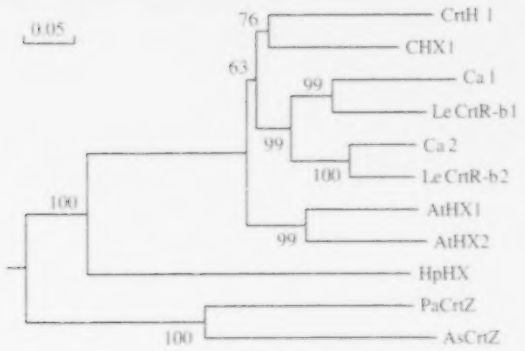


Fig. 5 Phylogenic relationship between the β-carotene hydroxylase of *Adonis aestivalis* (*CrtH1*) and those of other organisms. GenBank accession numbers of these sequences are the following: *Arabidopsis thaliana AtHX1* (AF370220) and *AtHX2* (NM_124636); *Citrus unshiu CHX1* (AF296158); *Lycopersicon esculentum LeCrtR-b1* (Y14809) and *LeCrtR-b2* (Y14810); *Capsicum annuum Ca1* (Y09225) and *Ca2* (Y09722); *Haematococcus pluvialis HpHX* (AF162276); *Alcaligenes sp. AsCrtZ* (D58422); *Pantoea agglomerans PaCrtZ* (M87280). The number of bootstrap is 40,000 which was performed to test the reliability of the branches. The *bar* indicates the scale for branch length

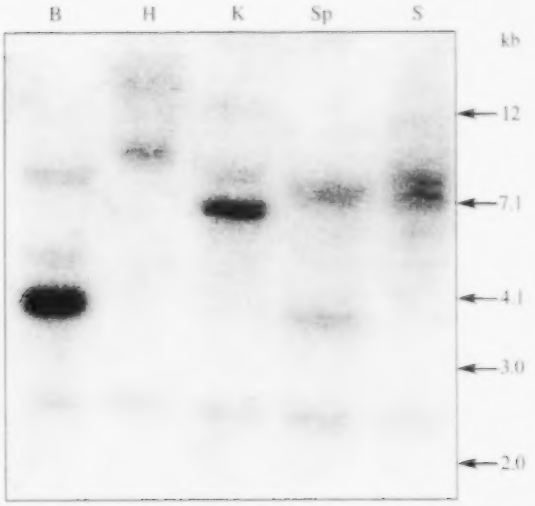


Fig. 6 Southern-blot analysis of the *CrtH1* gene of *Adonis aestivalis*. Approximately 10 μg of genomic DNA was digested with *Bam*HI (*B*), *Hind*III (*H*), *Kpn*I (*K*), *Spe*I (*Sp*) and *Sst*I (*S*) restriction endonucleases, none of which cuts within *CrtH1* cDNA sequence. Size markers (kb) are indicated on the right

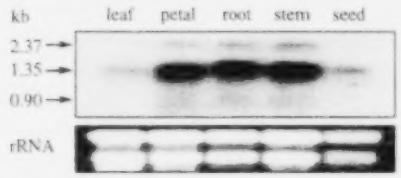


Fig. 7 Expression profile of *CrtH1* in different organs of *Adonis aestivalis*. Approximately 15 μg of total RNA was electrophoresed on 1.2% agarose/formaldehyde gel and blotted onto Hybond-XL membrane. Probe was the same as that used for Southern analysis in Fig. 6

CrtH1 converts β-carotene to β-cryptoxanthin and zeaxanthin in *E. coli*

Amino acid alignment showed that CrtH1 shared sequence similarity with β-carotene hydroxylases of other plants (Fig. 4). Therefore, we undertook to determine whether CrtH1 exhibited β-carotene hydroxylase activity. A functional assay was conducted in an *E. coli* strain possessing plasmid pBS-CrtH1 for the expression of the CrtH1 ORF and plasmid pACCAR16ΔcrtX, which expresses the genes required for the biosynthesis of β-carotene (Misawa et al. 1995). HPLC analysis to determine the carotenoid profiles of the *E. coli* strains showed that cells harboring plasmids pBS-CrtH1 and pACCAR16ΔcrtX produced β-carotene, β-cryptoxanthin and zeaxanthin (Fig. 8). Of total detectable carotenoids in triplicate samples, β-cryptoxanthin and zeaxanthin accounted for 24.5% (±0.4%) and 38.9% (±0.7%), respectively. As expected, the negative control *E. coli* strain harboring plasmids pBluescriptII KS (+) and pACCAR16ΔcrtX accumulated only β-carotene (Fig. 8). Compounds eluting at different peaks were identified by their retention time and absorption spectra compared to the corresponding authentic standards as well as by co-chromatography with authentic standards. This analysis confirms that *CrtH1* encodes an enzyme with β-carotene hydroxylase activity that converts β-carotene to zeaxanthin via β-cryptoxanthin (mono-hydroxylated β-carotene). The accumulation of a high level of β-cryptoxanthin in the *E. coli* expression system suggested asymmetric introduction of hydroxyl groups to the β-rings of β-carotene by the CrtH1 enzyme. This could be due to the possibility that CrtH1 expressed in *E. coli* may have a slightly altered configuration that confers a different mechanism for substrate recognition that allows for asymmetric hydroxylation of the β-rings of β-carotene. It is also possible that, in *E. coli*, CrtH1 could not form a stable dimer that is deemed necessary for the full function of β-carotene hydroxylases (Sun et al. 1996). For two-step carotenogenic enzymes, dimerization is considered essential for catalysis of the second reaction (Römer et al. 2002). CrtH1 protein may also be unstable in *E. coli*, and only a small number of dimers are formed at low cellular concentrations of CrtH1. This could explain the accumulation of high levels of mono-hydroxyl β-cryptoxanthin in the *E. coli* assay. The

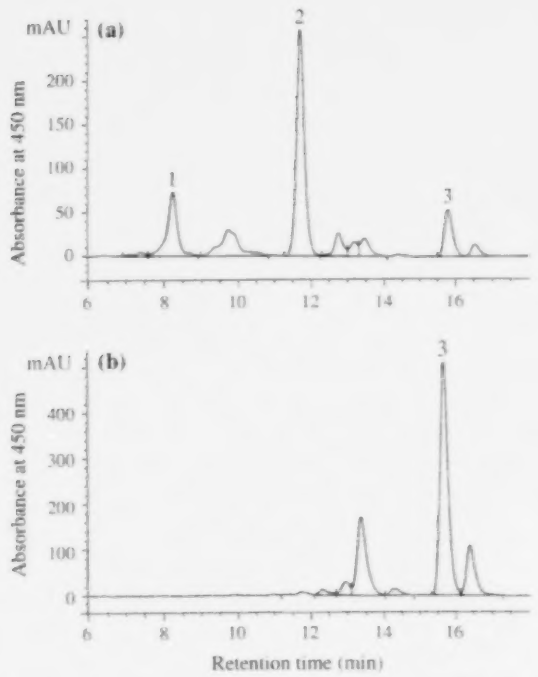


Fig. 8 HPLC profiles of carotenoids extracted from *E. coli* cells carrying plasmids. **a** pBS-CrtH1 and pACCAR16ΔcrtX. **b** empty cloning vector pBluescriptII KS (+) and pACCAR16ΔcrtX. Peaks numbered *1*, *2* and *3* correspond to zeaxanthin, β-cryptoxanthin and β-carotene, respectively

catalytic properties of CrtH1 are different from those of *A. thaliana* β-carotene hydroxylases which could convert β-carotene to ~90% zeaxanthin and ~6% β-cryptoxanthin (Sun et al. 1996; Tian and DellaPenna 2001) using similar functional assays in *E. coli*. The β-carotene hydroxylase CrtR from the cyanobacterium *Synechocystis* sp. PCC6803 also showed symmetric hydroxylation reaction towards the two β-rings of β-carotene in *E. coli* (Masamoto et al. 1998).

Expression of *CrtH1* in *A. thaliana* leads to the accumulation of violaxanthin

In plants, a key enzymatic step in the biosynthesis of lutein and β-carotene-derived xanthophylls is the hydroxylation reaction carried out by β-carotene hydroxylase. The aforementioned functional assay in *E. coli* demonstrated that CrtH1 exhibited β-carotene hydroxylase activity. To investigate the effectiveness of using CrtH1 to alter carotenoid biosynthesis in plants, a transformation construct for overexpressing this gene under the control of the seed-specific napin promoter was made, and used to transform wild type *A. thaliana* (wt) and the b1b2 mutant deficient for endogenous β-carotene hydroxylase activities (Tian et al. 2003). To assess the level of *CrtH1* expression in transgenic *A. thaliana*, Northern-blot analysis was conducted on total RNA isolated from immature siliques of select transgenic lines. The transgenic lines had significant levels of *CrtH1* expression, whereas no expression could be detected in untransformed control plants (Fig. 9). No obvious visible phenotypic differences were observed among *A. thaliana* wt, b1b2 mutant and transgenic lines expressing *CrtH1* (data not shown). Mature seeds were assessed for the presence and levels of carotenoids. HPLC traces of representative wt and b1b2 mutant lines expressing *CrtH1*, as well as those of wild type and b1b2 mutant are illustrated in Fig. 10, which shows the heterologous expression of *CrtH1* in *A. thaliana* caused an increase in the level of violaxanthin (peak 1). Table 1 shows HPLC quantification of carotenoids in seeds from representative *A. thaliana* lines. Decreases in the range of 15–78% in the amount of β-carotene were observed in most of the transgenic lines compared to untransformed plants; this is expected as a result of enhanced hydroxylation and conversion of β-carotene to xanthophylls. Expression of *CrtH1* in the wild type line resulted in 44–64% increase in lutein content, except for the BY275 and BY284 lines, which did not show any significant difference in lutein level. With the exception of the BY330 line, all b1b2 mutant lines expressing *CrtH1* showed increases in lutein levels ranging from14 to 72% as compared to b1b2 mutant. The hydroxylation of the β-ring of α-carotene is required for its conversion to lutein. Therefore, enhanced biosynthesis of lutein in lines expressing *CrtH1* would be expected if this gene encodes a β-carotene hydroxylase. It is noteworthy that β-carotene-derived xanthophylls continued to be detected in the b1b2 mutant, possibly as a result of functional overlap between β- and ε-hydroxylases (Tian et al. 2003). β-cryptoxanthin (mono-hydroxylated β-carotene), an intermediate in the conversion of β-carotene to zeaxanthin, was undetectable in wild type, but was detected in the b1b2 mutant as a result of incomplete hydroxylation of the β-rings of β-carotene. β-cryptoxanthin was also undetectable in wild type lines expressing *CrtH1* except in the BY293 line where trace amounts were detected. Levels of β-cryptoxanthin in b1b2 mutant lines expressing *CrtH1* were slightly reduced or undetectable as a result of its conversion to zeaxanthin and then violaxanthin. The only exception was the BY341 line, which showed approximately 41% increase in β-cryptoxanthin, presumably due to its inefficient hydroxylation to zeaxanthin. This result is in contrast to that obtained in the functional assay of CrtH1 in *E. coli* where β-cryptoxanthin accumulated at

Table 1 Concentrations of carotenoids in *Arabidopsis thaliana* seeds from select lines transformed with CrtH1 gene

Plant line	Violaxanthin μg/g dw	Lutein μg/g dw	Zeaxanthin μg/g dw	β-Cryptoxanthin μg/g dw	β-Carotene μg/g dw
wt	0.694 ± 0.056	13.380 ± 0.729	0.955 ± 0.051	UD	1.890 ± 0.504
wt+CrtH1					
BY275	3.186 ± 0.544	13.413 ± 3.354	0.776 ± 0.193	UD	1.009 ± 0.188
BY280	5.47 ± 0.342	21.631 ± 0.941	1.162 ± 0.036	UD	0.422 ± 0.033
BY281	6.162 ± 0.416	20.684 ± 0.623	1.067 ± 0.033	UD	0.4 ± 0.007
BY284	2.939 ± 0.199	12.678 ± 1.367	0.642 ± 0.100	UD	0.718 ± 0.081
BY287	7.572 ± 0.284	22.016 ± 0.483	1.077 ± 0.011	UD	0.591 ± 0.068
BY293	5.611 ± 0.216	21.49 ± 0.597	0.895 ± 0.034	0.098 ± 0.002	1.491 ± 0.142
BY294	3.975 ± 0.12	19.306 ± 0.95	0.883 ± 0.016	UD	1.015 ± 0.074
b1b2 mutant	0.881 ± 0.036	26.214 ± 2.690	0.711 ± 0.082	0.153 ± 0.014	8.238 ± 1.019
b1b2 mutant +CrtH1					
BY317	24.741 ± 2.37	42.523 ± 2.275	0.727 ± 0.023	UD	2.473 ± 0.439
BY330	5.522 ± 1.295	25.912 ± 4.857	0.544 ± 0.048	0.139 ± 0.043	6.41 ± 0.081
BY334	8.713 ± 1.092	34.809 ± 2.493	0.611 ± 0.039	0.156 ± 0.006	8.942 ± 0.88
BY341	11.005 ± 0.548	33.705 ± 2.331	0.526 ± 0.061	0.216 ± 0.075	5.78 ± 0.435
BY342	20.62 ± 0.688	45.199 ± 0.709	0.731 ± 0.025	UD	3.099 ± 0.228
BY346	15.111 ± 1.103	36.464 ± 1.591	0.584 ± 0.037	0.164 ± 0.013	3.262 ± 0.11
BY347	3.871 ± 0.163	30.08 ± 1.882	0.616 ± 0.014	0.148 ± 0.015	6.985 ± 0.515

UD undetectable, *dw* dry weight, Each value is the mean result from triplicate ±SD

a higher level. This indicates that CrtH1 may be more efficient at fully hydroxylating β-carotene rings in plants than in *E. coli*. Most of the wild type and b1b2 mutant lines expressing *CrtH1* showed either a slight decrease or no statistically significant change in levels of zeaxanthin when compared to untransformed wild type and b1b2 mutant. However, elevated levels of violaxanthin (at least a threefold increase) were observed in all *CrtH1* expressing lines when compared to their untransformed counterparts. In wild type lines expressing *CrtH1*, violaxanthin level increased by up to tenfold. Of all the b1b2 mutant plants expressing *CrtH1*, the BY317 line had the highest level of violaxanthin with a 27-fold increase compared to the b1b2 mutant. The lack of zeaxanthin accumulation combined with enhanced violaxanthin levels in transgenic lines is an indication that zeaxanthin epoxidase is active enough to convert excess zeaxanthin to violaxanthin in seeds of *A. thaliana*. In plants, there exists a mechanism involving a set of reactions in the xanthophyll cycle: the reversible interconversion of zeaxanthin and violaxanthin through the intermediate product antheraxanthin (Fig. 1; Davison et al. 2002), which rapidly optimizes the concentration of zeaxanthin (Demmig-Adams and Adams 2002). Therefore, more violaxanthin would be expected if the zeaxanthin biosynthesis was enhanced by β-carotene hydroxylase. The xanthophyll cycle helps to protect against light-induced damage by converting violaxanthin into zeaxanthin which participates in thermal dissipation of excessively absorbed light energy (Malkin and Niyogi 2002). It is noteworthy that seeds from b1b2 mutant expressing *CrtH1* had on average higher level of violaxanthin than those from wild

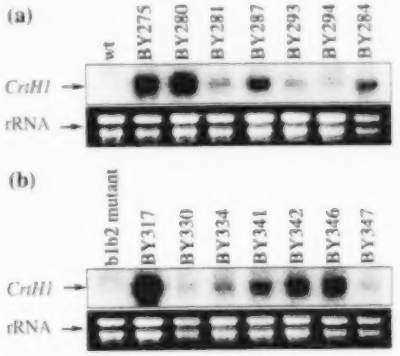


Fig. 9 **a**, **b** Northern analysis of *CrtH1* in immature siliques of transgenic *Arabidopsis thaliana*. **a** Wild type (wt) and wild type expressing *CrtH1* (BY275 to BY284) , **b** b1b2 mutant, and b1b2 mutant expressing *CrtH1* (BY317 to BY347). Approximately 10 μg of total RNA was electrophoresed on 1.2% agarose/formaldehyde gel and blotted onto Hybond-XL membrane. The blot was hybridized with a 350 bp fragment from the 5′-end of the *CrtH1* ORF

type plants expressing *CrtH1*. This might be due to the co-supression of the endogenous β-carotene hydroxylase in wild type lines expressing *CrtH1*, because of the high nucleotide homology between *CrtH1* and the ORFs of the two β-carotene hydroxylases of *A. thaliana* (AF370220 and NM_124636). Alignment of the three ORFs revealed an overall homology level of 72%, but a region of about 600 bp showed a homology of 89% (data not shown); a level of homology sufficient to result in co-suppression of the endogenous genes.

No correlation could be drawn between levels of accumulation of different species of carotenoids and

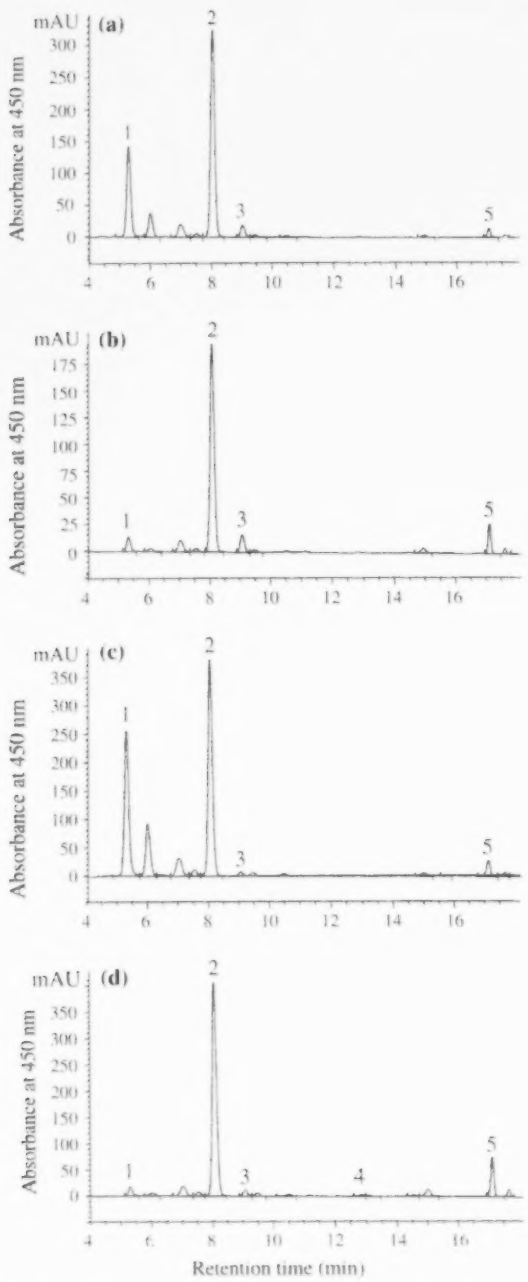


Fig. 10 HPLC profiles of carotenoids extracted from seeds of *Arabidopsis thaliana*. **a** Wild type expressing *CrtH1* (BY287line), **b** wild type, **c** b1b2 mutant expressing *CrtH1* (BY317line) and **d** b1b2 mutant. Peaks numbered *1*, *2*, *3*, *4* and 5 correspond to violaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene, respectively

levels of *CrtH1* expression in different transgenic *A. thaliana* lines. This suggests a differential role for *CrtH1* expression in the regulation of carotenoid biosynthesis in individual transformants. This also indicates that the β-carotene hydroxylase activity of CrtH1 may be constrained by factors such as substrate availability or feedback inhibition.

When a β-carotene hydroxylase gene was constitutively overexpressed in *A. thaliana*, the transformants were more tolerant to conditions of high light and high temperature (Davison et al. 2002). Also, transgenic tobacco overexpressing a bacterial β-carotene hydroxylase showed an enhanced UV photoprotection (Götz et al. 2002). Whether *A. thaliana* plants expressing *CrtH1* are more tolerant to stress conditions requires further investigation.

Conclusions

We present data supporting that *CrtH1* encodes a β-carotene hydroxylase in *Adonis aestivalis*. The predicted amino acid sequence of CrtH1 showed strong homology to β-carotene hydroxylases of other organisms. Features of the translated protein are consistent with a plastid-localized β-carotene hydroxylase. These features include a plastid transit peptide, transmembrane helices and conserved histidine motifs. Functional assays in *E. coli* showed that CrtH1 could convert β-carotene to its hydroxylated derivatives, β-cryptoxanthin and zeaxanthin. Expression of *CrtH1* gene in *A. thaliana* wild type and the b1b2 mutant, which has T-DNA knockouts in the two β-carotene hydroxylase genes, caused a marked increase in the level of violaxanthin. The *CrtH1* gene is a tool that may be useful in modifying the biosynthetic pathway of carotenoids in higher plants, especially cruciferous species, such as *A. thaliana* and Brassica oilseeds. As these species are evolutionarily distant from *A. aestivalis*, *CrtH1* would be expected to cause less co-suppression than its homologues from crucifers. Because of the economic importance of carotenoids, metabolic engineering of their biosynthesis has been explored in several plant species (Shewmaker et al. 1999; Ye et al. 2000; Römer et al. 2002; Dharmapuri et al. 2002; Ravanello et al. 2003; Ralley et al. 2004). Changes in carotenoid profiles in *A. thaliana* seeds caused by the expression of *CrtH1* provide information that may be useful in engineering carotenoid biosynthesis in related plant species.

Acknowledgments We thank Dr. Norihiko Misawa (Kirin Brewery Co. Ltd, Kanagawa, Japan) and Dr. Hartmut Linden (Universitat Konstanz, Germany) for the kind gift of plasmid pACCAR16ΔcrtX and Dr. Dean DellaPenna (Michigan State University, Michigan, USA) for generously providing the seeds of *Arabidopsis thaliana* b1b2 double knockout mutant. We are grateful to Drs. Isobel Parkin and Kevin Rozwadowski for critical

reading of the manuscript, and to Delwin Epp for technical help with HPLC analysis. Funding for this project was provided by the Saskatchewan Agriculture Development Fund.

References

Ausich RL (1997) Commercial opportunities for carotenoid production by biotechnology. Pure Appl Chem 69:2169–2173

Bouvier F, Keller Y, d'Harlingue A, Camara B (1998) Xanthophyll biosynthesis: molecular and functional characterization of carotenoid hydroxylases from pepper fruits (*Capsicum annuum* L.). Biochim Biophys Acta 1391:320–328

Carpenter CD, Simon AE (1998) Preparation of RNA. In: Martinez-Zapater JM, Salinas J (eds) Methods in molecular biology, vol. 82: *Arabidopsis* protocols. Humana Press, Totowa, NJ, pp 85–89

Church G, Gilbert W (1984) Genome sequencing. Proc Natl Acad Sci USA 81:1991–1995

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Cunningham FX Jr, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 49:557–583

Cunningham FX Jr, Gantt E (2005) A study in scarlet: enzymes of ketocarotenoid biosynthesis in the flowers of *Adonis aestivalis*. Plant J 41:478–492

Davison PA, Hunter CN, Horton P (2002) Overexpression of β-carotene hydroxylase enhances stress tolerance in *Arabidopsis*. Nature 418:203–206

Demmig-Adams B, Adams III WW (2002) Antioxidants in photosynthesis and human nutrition. Science 298:2149–2153

Dharmapuri S, Rosati C, Pallara P, Aquilani R, Bouvier F, Camara B, Giuliano G (2002) Metabolic engineering of xanthophyll content in tomato fruits. FEBS Lett 519:30–34

Fraser PD, Miura Y, Misawa N (1997) In vitro characterization of astaxanthin biosynthetic enzymes. J Biol Chem 272:6128–6135

Giuliano G, Bartley GE, Scolnik PA (1993) Regulation of carotenoid biosynthesis during tomato development. Plant Cell 5:379–387

Giuliano G, Aquilani R, Dharmapuri S (2000) Metabolic engineering of plant carotenoids. Trends Plant Sci 5:406–409

Götz T, Sandmann G, Römer S (2002) Expression of a bacterial carotene hydroxylase gene (*crtZ*) enhances UV tolerance in tobacco. Plant Mol Biol 50:129–142

Hirschberg J (1998) Molecular biology of carotenoid biosynthesis. In: Britton G, Liaaen-Jensen S, Pfander H (eds) Carotenoids, vol 3: biosynthesis and metabolism. Birkhaeuser Verlag, Berlin, pp 149–194

Hirschberg J (2001) Carotenoid biosynthesis in flowering plants. Curr Opin Plant Biol 4:210–218

Hundle BS, O'Brien DA, Beyer P, Kiemig H, Hearst JE (1993) In vitro expression and activity of lycopene cyclase and β-carotene hydroxylase from *Erwinia herbicola*. FEBS Lett 315:329–334

Johnson EA, Schroeder WA (1996) Microbial carotenoids: downstream processing biosurfactants. Adv Biochem Eng Biotechnol 53:119–178

Kim IJ, Ko KC, Kim CS, Chung WI (2001) Isolation and characterization of cDNAs encoding β-carotene hydroxylase in *Citrus*. Plant Sci 161:1005–1010

Linden H (1999) Carotenoid hydroxylase from *Haematococcus pluvialis*: cDNA sequence, regulation and functional complementation. Biochim Biophys Acta 1446:203–212

Malkin R, Niyogi K (2002) Photosynthesis. In: Buchanan BB, Gruissem W, Jones RL (eds) Biochemistry and molecular biology of plants. Science Press, Beijing, pp 579–580

Masamoto K, Misawa N, Kaneko T, Kikuno R, Toh H (1998) β-Carotene hydroxylase gene from cyanobacterium *Synechocystis* sp. PCC6803. Plant Cell Physiol 39:560–564

Mayne ST (1996) Beta-carotene, carotenoids and disease prevention in humans. FASEB J 10:690–701

Misawa N, Kajiwara S, Kondo K, Yokoyama A, Satomi Y, Saito T, Miki W Ohtani T (1995) Canthaxanthin biosynthesis by the conversion of methylene to keto groups in a hydrocardon β-carotene by a single gene. Biochem Biophys Res Commun 209:867–876

Moehs C, Tian L, Osteryoung KW, Dellapenna D (2001) Analysis of carotenoid biosynthetic gene expression during marigold petal development. Plant Mol Biol 45:281–293

Müller-Moulé P, Havaux M, Niyogi KK (2003) Zeaxanthin deficiency enhances the high light sensitivity of an ascorbate-deficient mutant of *Arabidopsis*. Plant Physiol 133:748–760

Ralley L, Enfissi EMA, Misawa N, Schuch W, Bramley PM, Fraser PD (2004) Metabolic engineering of ketocarotenoid formation in higher plants. Plant J 39:477–486

Rask L, Ellerstrom M, Ezcurra I, Stalberg K, Wycliffe P (1998) Seed-specific regulation of the napin promoter in *Brassica napus*. J Plant Physiol 152:505–599

Ravanello MP, Ke D, Alvarez J, Huang B, Shewmaker CK (2003) Coordinate expression of multiple bacterial carotenoid genes in canola leading to altered carotenoid production. Metab Eng 5:255–263

Rissler HM, Pogson BJ (2001) Antisense inhibition of the beta-carotene hydroxylase enzyme in *Arabidopsis* and the implications for carotenoid accumulation, photoprotection and antenna assembly. Photosynth Res 67:127–137

Römer S, Lubeck J, Kauder F, Steiger S, Adomat C, Sandmann G (2002) Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. Metab Eng 4:263–272

Seybold A, Goodwin TW (1959) Occurrence of astaxanthin in the flower petals of *Adonis annua* L. Nature 184:1714–1715

Shewmarker CK, Sheehy JA, Daley M, Colburn S, Ke DY (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. Plant J 20:401–412

Sun Z, Gantt E, Cunningham FX Jr (1996) Cloning and functional analysis of the β-carotene hydroxylase of *Arabidopsis thaliana*. J Biol Chem 271:24349–24352

Taylor M, Ramsay G (2005) Carotenoid biosynthesis in plant storage organs: recent advances and prospects for improving plant food quality. Physiol Plant 124:143–151

Tian L, DellaPenna D (2001) Characterization of a second carotenoid β- hydoxylase gene from *Arabidopsis* and its relationship to the LUT1 locus. Plant Mol Biol 47:379–388

Tian L, DellaPenna D (2004) Progress in understanding the origin and functions of carotenoid hydroxylases in plants. Arch Biochem Biophys 430:22–29

Tian L, Magallanes-Lundback M, Musetti V, DellaPenna D (2003) Function analysis of β- and ε-ring carotenoid hydroxylases in *Arabidopsis*. Plant Cell 15:1320–1332

Umeno D, Tobias AV, Arnold FH (2005) Diversifying carotenoid biosynthetic pathway by directed evolution. Microbiol Mol Biol Rev 69:51–78

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (*b*-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287:303–305

Transgenic Res
DOI 10.1007/s11248-007-9131-x

ORIGINAL PAPER

Enhancing the carotenoid content of *Brassica napus* seeds by downregulating lycopene epsilon cyclase

Bianyun Yu · Derek J. Lydiate · Lester W. Young · Ulrike A. Schäfer · Abdelali Hannoufa

Received: 21 June 2007 / Accepted: 13 August 2007
© Springer Science+Business Media B.V. 2007

Abstract The accumulation of carotenoids in higher plants is regulated by the environment, tissue type and developmental stage. In *Brassica napus* leaves, β-carotene and lutein were the main carotenoids present while petals primarily accumulated lutein and violaxanthin. Carotenoid accumulation in seeds was developmentally regulated with the highest levels detected at 35–40 days post anthesis. The carotenoid biosynthesis pathway branches after the formation of lycopene. One branch forms carotenoids with two β rings such as β-carotene, zeaxanthin and violaxanthin, while the other introduces both β- and ε-rings in lycopene to form α-carotene and lutein. By reducing the expression of lycopene ε-cyclase (*ε-CYC*) using RNAi, we investigated altering carotenoid accumulation in seeds of *B. napus*. Transgenic seeds expressing this construct had increased levels of β-carotene, zeaxanthin, violaxanthin and, unexpectedly, lutein. The higher total carotenoid content resulting from reduction of *ε-CYC* expression in seeds suggests that this gene is a rate-limiting step in the carotenoid biosynthesis pathway. *ε-CYC* activity and carotenoid production may also be related to fatty acid biosynthesis in seeds as transgenic seeds showed an overall decrease in total fatty acid content and minor changes in the proportions of various fatty acids.

Keywords Carotenoids · Lycopene ε-cyclase · *Brassica napus* · Seeds · RNAi silencing

B. Yu · D. J. Lydiate · L. W. Young · U. A. Schäfer · A. Hannoufa (✉)
Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, SK, Canada S7N 0X2
e-mail: hannoufaa@agr.gc.ca

Present Address:
L. W. Young
Department of Plant Sciences, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK, Canada S7N 5A8

Introduction

Carotenoids comprise a large group of secondary metabolites that are natural pigments present in most higher plants (Demmig-Adams et al. 1996; Vishnevetsky et al.1999; Cuttriss and Pogson 2004; Howitt and Pogson 2006). They are essential components of photosynthetic membranes and provide photoprotection against light damage by channeling excess energy away from chlorophyll (Bassi et al. 1993; Young 1993; Kuhlbrandt et al. 1994; Bartley and Scolnik 1995; Demmig-Adams and Adams 2002). In addition, carotenoids act as membrane stabilizers (Demmig-Adams et al. 1996) and are also precursors in abscisic acid (ABA) biosynthesis (Rock and Zeevaart 1991; Lindgren et al. 2003; Howitt and Pogson 2006). Carotenoids are synthesized and accumulated in the plastids of higher plants (Cunningham and Gantt 1998). Chloroplasts store carotenoids in thylakoid membranes associated with

light harvesting (Peter and Thornber 1991; Cunningham and Gantt 1998), while chromoplasts may store high levels of carotenoids in membranes, oil bodies, or other crystalline structures within the stroma (Kirk and Tilney-Bassett 1978; Cunningham and Gantt 1998; Howitt and Pogson 2006).

Carotenoids are derived from the isoprenoid pathway with the first committed step being the condensation of two geranylgeranyl diphosphates to form phytoene (Taylor and Ramsay 2005). Phytoene then undergoes four sequential desaturation reactions to form lycopene (Cunningham and Gantt 1998). In higher plants the cyclization of lycopene with lycopene β- and ε-cyclases is the branch-point in carotenoid biosynthesis (Cunningham and Gantt 1998; Fig. 1). On one branch a single enzyme, lycopene β-cyclase (β-CYC), introduces a β-ring at both ends of lycopene to form β-carotene in a two-step reaction. The first dedicated reaction in the other branch of the pathway, leading to lutein, requires both β-CYC and lycopene ε-cyclase (ε-CYC) to introduce one β- and one ε-ring into lycopene to form α-carotene (Pogson et al. 1996; Cunningham and Gantt 1998; Howitt and Pogson 2006). In contrast, a novel ε-CYC from the cyanobacterium *Prochlorococcus marinus* MED4, was shown to catalyze the simultaneous formation of α-, β- and ε-carotene (Stickforth et al. 2003). Carotenoids with two ε-rings are rare in plants and algae (Goodwin 1980); however an ε-CYC of romaine lettuce uniquely adds two ε-rings to lycopene to form lactucaxanthin (Cunningham and Gantt 2001). A set of reactions in plants, the xanthophyll cycle, rapidly optimizes the concentration of zeaxanthin and violaxanthin in the cell through the action of zeaxanthin epoxidase and violaxanthin de-epoxidase via antheraxanthin (Demmig-Adams and Adams 2002). In *Arabidopsis thaliana*, nine *cis*-epoxycarotenoid dioxygenase (NCED) enzymes cleave the *cis*-isomers of violaxanthin and neoxanthin to form xanthoxin, which is the precursor of ABA (Nambara and Marion-Poll 2005).

Carotenoids are widely used in the food and cosmetics industries (Fraser and Bramley 2004; Taylor and Ramsay 2005; Botella-Pavia and Rodriguez-Concepción 2006), and their importance to human health has been well documented (Bartley and Scolnik 1995; Mayne 1996; Demmig-Adams and Adams 2002; Krinsky and Johnson 2005). For example, β-carotene is the precursor of vitamin A (Lakshman and Okoh 1993) and lutein and zeaxanthin provide protection against macular degeneration (Landrum and Bone 2004). Vitamin A (retinol) deficiency in humans results in symptoms ranging from night blindness to total and irreversible blindness (Ye et al. 2000). The dietary consumption of foods rich in provitamin A (β-carotene) avoids

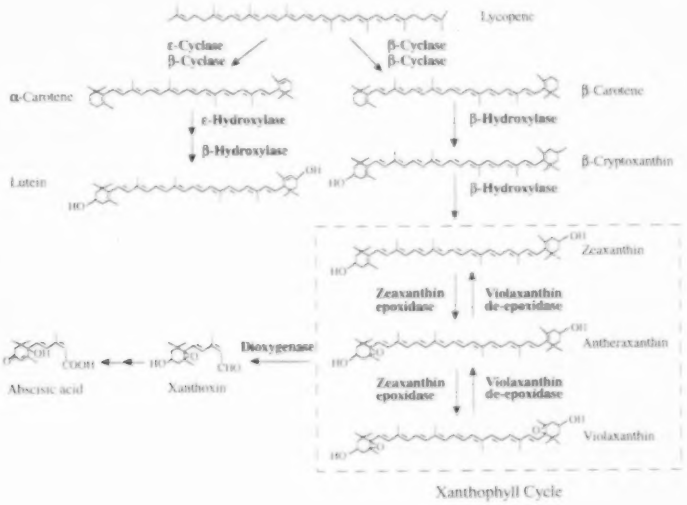


Fig. 1 The carotenoid biosynthesis pathway in plants

deficiency. Lutein and zeaxanthin also help protect the eye by absorbing potentially harmful blue light radiation (Krinsky and Johnson 2005). Carotenoid levels in many crops used in human and animal diets are inadequate and metabolic engineering provides a promising tool to fortify plants with these essential nutrients. Several examples demonstrating metabolic engineering approaches to increase carotenoid concentrations in plants have been reported (reviewed in Botella-Pavia and Rodriguez-Concepcion 2006). Overexpression of a bacterial phytoene synthase (PSY) in a seed-specific manner in *Brassica napus* resulted in a 50-fold increase in carotenoid concentrations (Shewmaker et al. 1999). Tuber-specific expression of a bacterial *PSY* in potato enhanced levels of both β-carotene and lutein (Ducreux et al. 2005). Overexpression of the endogenous *PSY* in the seeds of *A. thaliana* resulted in a 43-fold average increase in the level of β-carotene (Lindgren et al. 2003) while expression of *A. thaliana* lycopene β-CYC led to a significant increase in β-carotene content in tomato fruits (Rosati et al. 2000). Expression of the daffodil *PSY* and a bacterial phytoene desaturase (CrtI) in rice resulted in the production of β-carotene, lutein and zeaxanthin in the endosperm (Ye et al. 2000). Tuber-specific silencing of lycopene epsilon cyclase (*LCY-e*) or β-carotene hydroxylase (*CHY1* and *CHY2*) increased the levels of β-carotene and total carotenoids in potato tuber (Diretto et al. 2006; Diretto et al. 2007).

In this report, we show differential accumulation of carotenoid compounds in different *B. napus* organs and in seeds at different developmental stages. In an attempt to enhance the level of β-carotene in the seed of *B. napus*, we downregulated the expression of *ε-CYC* using RNAi. This strategy was aimed at reducing the level of lutein present in seeds and diverting lycopene to β-carotene biosynthesis only. Using our RNAi system to repress *ε-CYC* led to increased levels of β-carotene, lutein, zeaxanthin and violaxanthin in *B. napus* seeds.

Materials and Methods

Vector construction for RNAi and plant transformation

Two *B. napus* expressed sequence tags (EST), CL1624 and CL1622, homologous to the 5′- and 3′-ends, respectively, of the *A. thaliana ε-CYC* (NM_125085) were identified from the *B. napus* EST collection held at the Saskatoon Research Centre (http://www.brassica.ca). These two ESTs were used to generate RNAi constructs specific to the 5′ and 3′ ends of *ε-CYC*. The *ε-CYC*-specific 5′ and 3′ fragments were amplified by the polymerase chain reaction (PCR). Primers P1 and P2 having built-in *Spe*I and *Asc*I or *Bam*HI and *Swa*I sites, respectively (Table 1) were used to generate the 5′-end fragment of 352 bp, and a 410 bp from the 3′-end was amplified using primers P3 and P4. Single palindromic repeats of the 5′ and 3′-end PCR products were inserted around a 300 bp spacer of β-glucuronidase in pGSA1285 vector (Fig. 2) (CAMBIA, Canberra, ACT, Australia). The resulting RNAi vectors were designated 710-422 for the 5′-end fragment and 710-423 for the 3′-end fragment.

Growth conditions and plant transformation

Cotyledon explants of *B. napus* doubled haploid line DH12075 were used for *Agrobacterium tumefaciens* (GV3101pMP90)-mediated transformation (Moloney et al. 1989). *B. napus* plants were grown in a controlled environment greenhouse (16 h light/8 h dark, 20°C/17°C). Only those plants shown to be transgenic as determined by PCR to assess the presence of the transgene were subjected to further analysis. The primers used were P2 and P5 for construct 710-422, and P4 and P5 for construct 710-423 (Table 1).

DNA isolation and Southern blot analysis

Total genomic DNA was isolated from leaves of *B. napus* using the DNeasy Plant Mini Kit (Qiagen, Mississauga, Canada). Approximately 10 μg of genomic DNA was digested with *Bam*HI, *Eco*RI, *Eco*RV, *Sal*I, *Spe*I and *Sst*I, separated on a 0.8% agarose gel and transferred onto Hybond-XL nylon membrane (Amersham Biosciences, Quebec, Canada). The membrane was probed with a 352 bp *B. napus ε-CYC*-specific fragment amplified by PCR using primers P6 and P7 from the leaf cDNA. Hybridization was performed using Church buffer (Church and Gilbert 1984) at 61°C for 22 h. The filter was washed

Table 1 List of primers used in the experimental procedures

Primer name	Primer sequence	Notes
P1	5′-cgactagtggcgcgccGAGGTTTTCGTCTCCG-3′	Restriction sites of *Spe*I and *Asc*I were indicated with lower case
P2	5′-cgggatccatttaaatCATCCATGTCTTTGTTCTG-3′	Restriction sites of *Bam*HI and *Swa*I were indicated with lower case
P3	5′-cgactagtggcgcgccCAGAAAGGAAACGACAA-3′	Restriction sites of *Spe*I and *Asc*I were indicated with lower case
P4	5′-cgggatccatttaaatCAATCTTCTAAGGCACGC-3′	Restriction sites of *Bam*HI and *Swa*I were indicated with lower case
P5	5′-CAATCCCACTATCCTTCGCAAGACCC-3′	
P6	5′-GAGGTTTTCGTCTCCG-3′	
P7	5′-CATCCATGTCTTTGTTCTG-3′	
P8	5′-CTTAGACAAGCGGCTTTGGTGAACA-3′	
P9	5′-ACTGAGCTCGGTGACGCCTTTCTC-3′	
P10	5′-CGAGATGCTGACATGGCCAGAGAAA-3′	
P11	5′-AGTGGCAAACACATAAGCGTCTCCT-3′	
P12	5′-AGAGAGGATGGTTGCGAGGCTGAA-3′	
P13	5′-AACAGCCTCGACGACAAGAAACCA-3′	
P14	5′-CACCAGAAAGGAAACGACAAAGAGCA-3′	
P15	5′-TTCTCTCAATCTTCTAAGGCACGCAC-3′	
P16	5′-GAGGTTTTCGTCTCCG-3′	
P17	5′-TTCTCTCAATCTTCTAAGGCACGCAC-3′	
P18	5′-TGAAAGATGGCCGATGGTGAGGA-3′	
P19	5′-CCGTCTCCAGCTCTTGCTCGTAGT-3′	
P20	5′-TGAAAGATGGCCGATGGTGAGGA-3′	
P21	5′-CACACTCACCACCACGAACCAGAAG-3′	

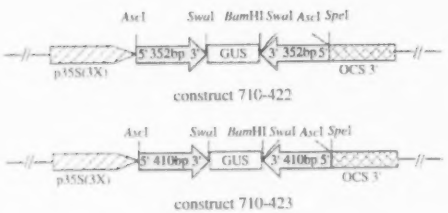


Fig. 2 Diagram of the RNAi constructs 710–422 and 710–423. Sequences were PCR amplified from the 5′ and 3′ ends of a *B. napus* lycopene ε-cyclase EST and used to generate the RNAi constructs

twice in 2 × SSC, 0.1% SDS for 10 min at 61°C and twice more in 0.2 × SSC, 0.1% SDS for 10 min at 61°C. X-ray film was then exposed to the filter with the aid of intensifying screen.

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from leaves, flower petals, roots and seeds at different developmental stages as described by Carpenter and Simon (1998), with some modifications. About 100 mg of ground tissue was extracted with 600 µl of RNA extraction buffer (0.2 M Tris–HCl, pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% SDS) and an equal volume of Tris–HCl buffered phenol (pH 7.9). Extraction was repeated twice with phenol and followed once with chloroform. Approximately 1/4 volume of 10 M LiCl was added to the decanted aqueous layer, mixed well, stored at 4°C overnight and then centrifuged at 14,000*g* for 20 min. The pellet was resuspended in 0.3 ml of DEPC-treated dH_2O, to which 30 µl of 3 M sodium acetate (pH 5.3) and 0.7 ml of 95% ethanol were added. The mixture was chilled at −70°C for

10 min and then centrifuged at 14, 000*g* for 20 min. The pellet was washed and resuspended in 20 µl of DEPC-treated dH_2O.

Total RNA was used for one-step semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis of *PSY*, *PDS*, *β-CYC* and *ε-CYC* gene expression. This method was chosen considering the low abundance of many carotenoid biosynthetic gene steady state mRNAs (Giuliano et al. 1993). Primers spanning introns were designed for each gene except *β-CYC*, which does not have an intron, to distinguish between products amplified from cDNA and genomic DNA (Table 1). For *β-CYC*, a negative control reaction was included in which the RT-PCR reaction lacked reverse transcriptase. Detection of actin mRNA was used as internal standard for RNA levels; two different sized actin amplicons were used to avoid co-migration with the target gene during gel electrophoresis: a 1178 bp actin fragment was co-amplified with *PSY*, while a 700 bp actin fragment was used with *PDS*, *β-CYC* and *ε-CYC*. For analysis of *ε-CYC* gene expression in developing seeds of transgenic plants, a 1.8 kb *ε-CYC* fragment and a 1.178 kb actin fragment were co-amplified. For analysis of *ε-CYC* gene expression in other tissues, 0.418 kb *ε-CYC* fragment and a 0.700 kb actin fragment were co-amplified. The primers used in the RT-PCR reactions (Table 1) were P8 and P9 for *PSY*; P10 and P11 for *PDS*; P12 and P13 for *β-CYC*; P14 and P15 for *ε-CYC*; P16 and P17 for *ε-CYC* (1.8 Kb); P18 and P19 for actin (0.700 kb); and P20 and P21 for actin (1.178 kb). RT-PCR co-amplification of the internal standard actin gene and test gene fragments was performed using the SuperScript One-Step RT-PCR System (Invitrogen, Burlington, Canada). Approximately 180 ng of DNase I (Amplification Grade, Invitrogen, Burlington, ON, Canada)-treated total RNA was used in a 25 µl RT-PCR reaction mixture. Reverse transcription was performed at 45°C for 30 min, followed by PCR amplification using an initial denaturation at 94°C for 4 min, then 26 cycles at 94°C (30 s), 55°C (30 s), 72°C (extension time varied depending on target gene) and a final extension at 72°C for 5 min. The RT-PCR products were separated on a 1.0% agarose gel and transferred to Hybond-XL membrane (Amersham Biosciences, QC, Canada). The blots were probed with [α-^{32}P]dCTP labelled gene-specific fragment. The ethidium bromide-stained gel photograph was used for the internal control gene, actin.

Extraction of carotenoids from *B. napus* seeds and HPLC analysis

Approximately 200 mg of seed in 3 ml extraction solvent (hexane/acetone/ethanol, 50/25/25) were pulverised by rapidly shaking for 30 min in a scintillation vial containing a steel rod (adapted from Shewmaker et al. 1999). The sample was centrifuged for 10 min at 1,800*g* and the supernatant collected. The pellet was washed with another 3 ml extraction solvent and the supernatant collected and pooled. The solvent was removed by evaporation at room temperature under a stream of nitrogen gas. Triacyl glycerides were saponified in the residue by heating at 80°C for 1 h in 5 ml methanolic-KOH (10% w/v KOH in methanol:water [80:20 v/v]). Carotenoids and aqueous compounds were partitioned using 2 ml H_2O and 3 ml petroleum ether. The ether phase and two 3 ml ether washes were collected, pooled and the solvent evaporated at room temperature under a nitrogen gas stream. The residue was resuspended in 200 µl of acetonitrile/methylene chloride/methanol (50/40/10 [v/v]) with 0.5% (w/v) butylated hydroxytoluene and filtered through a 0.2 µm pore size nylon syringe filter into an HPLC sample vial. The extract was immediately analysed using HPLC. Aliquots of 20 µl were loaded onto a 4.6 µm × 250 mm reverse-phase C_{30} YMC "Carotenoid Column" (Waters Ltd, Mississauga, ON, Canada) at 35°C. Mobile phases consisted of methanol (A) and *tert*-methyl butyl ether (B). A linear gradient starting at 95% A and 5% B, proceeding to 35% A and 65% B over 25 min and a flow rate of 1.2 ml min^{-1} was used for elution. Compounds in the eluate were monitored at 450 nm using a photodiode array. Peaks were identified by their retention time and absorption spectra compared to those of known standards (CaroteNature, Switzerland). Quantification of carotenoids was conducted using curves constructed with authentic standards.

Fatty acid analysis

The gas chromatography method described by Young et al. (2006) was used to determine fatty acid concentration and profile. Briefly, triplicate samples of approximately 30 mg of seeds were

homogenised in hexane containing 0.938 mg ml^{-1} heptadecanoic acid methyl ester (HAME; Sigma-Aldrich, Oakville, ON, Canada) as an internal standard. Lipids were transesterified in 6.7% sodium methoxide for 30 min and the solution neutralised in 10% citric acid. The hexane layer was filtered through a 0.45 μm PTFE syringe filter and a 1:20 dilution made. One microlitre of diluted methyl ester solution was injected in a DBwax column (10 m long, 0.1 mm ID, 0.2 μm film, Agilent Technologies Canada, Mississauga, ON, Canada) in a Hewlet Packard 6890 gas chroamatograph. Inlet temperature was set at 240°C, with hydrogen carrier gas and a 1/20 split, using nitrogen makeup gas. Column temperatures started at 150°C, ramped to 220°C at 50°C min^{-1} and were maintained for 7 min. Column pressure started at 50 psi at insertion and dropped to approximately 35 psi after 2 min. Fatty acid methyl esters were detected using a flame ionisation detector.

Microarray analysis

Microarray analysis of gene expression was conducted on developing seeds of wild type DH12075 and *ε*-CYC-RNAi line BY351. Ambion AminoAllyl MessageAmp II aRNA amplification kit was used for RNA amplification and labelling according to the manufacture's instructions (Austin, TX, USA). Cy-Dye Post-labelling reactive dye pack was purchased from Amersham (GE healthcare, Baie d'Urfe, QC, Canada). Initial data processing and analysis were performed in BASE database (http://www.base.thep.lu.se). *B. napus* 15 K oligo arrays were used.

Results

Carotenoid profiles of leaves, petals and developing seeds of *B. napus*

The carotenoid profiles of *B. napus* leaves, petals and developing seeds were determined using HPLC analysis. In leaves, lutein, *β*-carotene, violaxanthin and *β*-cryptoxanthin account for 43.30%, 44.16%, 11.46% and 0.84% of total carotenoids, respectively (Table 2). The levels (relative to total carotenoids) of violaxanthin (30.34%) and *β*-cryptoxanthin (8.85%) in the petals were higher than in the leaves, but the level of *β*-carotene (13.79%) was lower. We also observed that the profiles of carotenoids accumulating in the seed varied depending on the developmental stage (Table 2). The highest level of violaxanthin was detected in seeds 15–20 days post anthesis (DPA) and it gradually decreased as the seed matured. Seeds 35–40 DPA had the highest levels of lutein and *β*-carotene, with much lower concentrations observed in fresh mature and dry mature seeds. Trace amounts of zeaxanthin were detected in seeds 15–20 DPA to 35–40 DPA, but it was undetectable in fresh mature and dry mature seeds. *β*-cryptoxanthin, which is rapidly converted to zeaxanthin, was detectable only in seeds 15–20 DPA.

Expression profiles of genes in the carotenoid biosynthesis pathway

Semi-quantitative RT-PCR analysis was used to determine the transcript levels of some carotenoid biosynthesis genes in the different organs of *B. napus* (Fig. 3). Primers spanning intron regions were

Table 2 Concentrations of carotenoid compounds in leaves, petals and developing seeds of *B. napus* DH12075

Tissue	*β*-Carotene	Lutein	Violaxanthin	Zeaxanthin	*β*-Cryptoxanthin
Leaf	106.91 ± 13.63	104.83 ± 2.92	27.74 ± 1.81	0.59 ± 0.02	2.03 ± 0.11
Petal	6.74 ± 0.51	21.75 ± 0.92	14.84 ± 1.25	1.23 ± 0.17	4.33 ± 0.27
15–20 DPA seed	6.30 ± 0.22	7.47 ± 0.25	3.30 ± 0.24	0.09 ± 0.005	0.13 ± 0.004
35–40 DPA seed	19.23 ± 0.71	21.22 ± 0.30	2.09 ± 0.15	0.26 ± 0.04	UD
Fresh mature seed	0.06 ± 0.005	3.14 ± 0.26	0.09 ± 0.01	UD	UD
Dry mature seed	0.49 ± 0.28	3.30 ± 0.49	UD	UD	UD

Values expressed as μg/g FW; UD, undetectable; FW, fresh weight; DPA, days post-anthesis

Each value is the mean result from triplicate ± SD

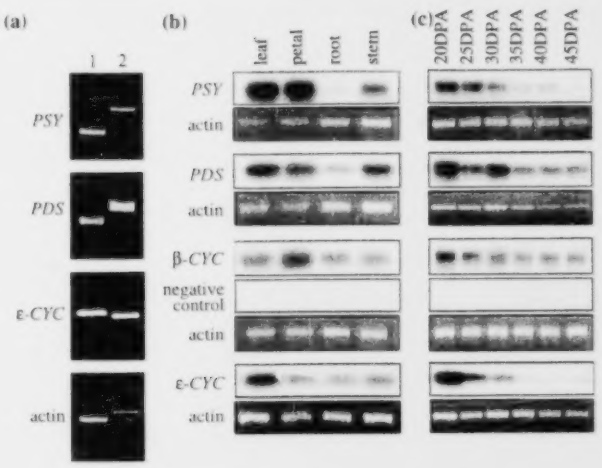


Fig. 3 Expression profiles of carotenoid biosynthesis genes in different organs, and in developing seeds of *B. napus*. (**a**) RT-PCR fragment amplified from templates of cDNA (1) and genomic DNA (2). (**b**) gene expression in different organs of *B. napus* relative to a co-amplified actin internal control. (**c**) gene expression in developing *B. napus* seeds relative to a co-amplified actin internal control. *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *β-CYC*, lycopene, *β*-cyclase; *ε-CYC*, lycopene *ε*-cyclase; DPA, days post-anthesis

designed for each gene, except for the intron-free *β-CYC*, to allow PCR products amplified from residual genomic DNA and target cDNA to be distinguished (Fig. 3a).

RT-PCR analysis revealed that *PSY*, *PDS*, and *ε-CYC* genes were highly expressed in leaves, petals and stems relative to weaker expression in roots (Fig 3b). The expression of *β-CYC* was highest in petals, and weaker in all other organs studied. Expression of these genes was also analyzed in developing seeds ranging from 20 to 45 DPA. During seed development, the expression of *PSY*, *PDS*, *β-CYC*, and *ε-CYC* was generally highest in early stages, i.e. up to 30 DPA, but decreased thereafter (Fig. 3c). Although expression of *PDS* and *β-CYC* declined during the later stages of seed development, the drop in relative expression levels appeared to be less compared to *PSY* and *ε-CYC*.

Silencing of *ε-CYC* increased levels of β-carotene and lutein

Two RNAi constructs, 710-422 and 710-423, were made to the 5′ and 3′ ends of *B. napus ε-CYC* and used to transform *B. napus* DH12075 line. RNA from the 25 DPA developing seeds of two transgenic lines, BY351 (expressing p710-422) and BY371 (expressing p710-423), were subjected to RT-PCR analysis to determine the expression levels of *ε-CYC* and other carotenoid biosynthesis genes, namely *PSY*, *PDS* and *β-CYC* (Fig. 4). Only the expression of *ε-CYC* and *PSY* was reduced in transgenic lines relative to the untransformed wild type control DH12075. Expression of PDS and *β-CYC* in seeds appeared to be unaltered by the expression of the RNAi construct at 25 DPA.

Visual observation of the color of carotenoid extracts from the mature seeds of *ε-CYC* silenced lines and the untransformed control DH12075 suggested that significant changes in carotenoid content had occurred (Data not shown). This was confirmed by using HPLC analysis to determine carotenoid profiles in mature seeds of transgenic and untransformed DH12075 plants. Seeds of the 10 transgenic lines tested had 2.6 to 41.7-fold higher concentrations of total carotenoids than those observed in DH12075. In DH12075 seeds, *β*-carotene and lutein, both of which are derived from lycopene, were the two main carotenoid compounds present (Table 3). However, *β*-carotene concentrations were at least 5.8-fold higher in the *ε-CYC* silenced lines than DH12075, with the greatest amount observed in line BY269 (185-fold). Unexpectedly, lutein concentrations were 1.9–22-fold greater in the transgenic lines than in DH12075. The ratio of *β*-carotene to lutein approximately doubled in the seeds of most transgenic lines, although 4.8, 4.9 and 8-fold in the relative amounts of *β*-carotene to lutein were observed in lines BY223, BY365 and BY269, respectively. Violaxanthin,

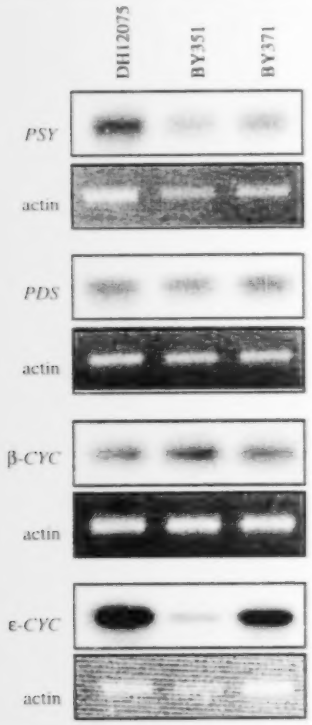


Fig. 4 Gene expression in developing seeds of DH12075 and *ε-CYC* RNAi lines, BY351 and 371. *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *β-CYC*, lycopene *β*-cyclase; *ε-CYC*, lycopene *ε*-cyclase

zeaxanthin and *β*-cryptoxanthin were undetectable in DH12075 seeds, but were detected in the 10 transgenic lines with the exception of *β*-cryptoxanthin in lines BY351, BY58 and BY371. Interestingly, statistically significant differences in carotenoid profiles were not observed in the leaves of transgenic plants compared to untransformed DH12075 even though the CaMV 35S constitutive expression promoter was used (data not shown).

Silencing of *ε-CYC* has minimal impact on fatty acid profiles

Of the ten transgenic lines tested, eight had lower concentrations of fatty acids than DH12075 (Table 4). For these eight lines the range of fatty acid content ranged from 22.3% to 31.1% of fresh weight (FW). The reductions in fatty acid content were not correlated with increased carotenoid levels, nor were there any patterns associated with the two constructs or generation of seeds used in the analysis.

The amount of palmitic acid in the transgenic seeds increased compared with DH12075, except for BY223 (Table 4). The concentrations of oleic acid and eicosanoic acid decreased compared with DH12075, except for oleic acid in BY371. Overall, the magnitude of the changes to the relative concentrations of fatty acids was small, except for the minor unidentified C18 compound.

Discussion

Our results showed that developing green seeds had higher *β*-carotene concentrations than mature seeds in wild type DH12075. This last observation was of particular interest since the purpose of this work was to investigate means to produce mature seeds with higher concentrations of *β*-carotene. Significant increases in carotenoid concentrations could be realised by preventing carotenoid reduction during seed maturation. However, preventing carotenoid breakdown or conversion to other compounds may require that the normal developmental changes that occur to plastids during seed maturation and desiccation be altered. These changes may result in abnormal plastid recovery and behaviour during dormancy and germination, with concomitant changes in seedling vigour (Lindgren et al. 2003). Therefore, rather than blocking catabolism of carotenoids, we tried to divert carotenoid production during development to produce seeds with higher accumulated levels of *β*-carotene.

Several attempts have been successfully made to engineer higher *β*-carotene in crop plants. One approach is to upregulate the supply of their precursor-phytoene (Shewmaker et al. 1999; Ducreux et al. 2005). Another strategy to increase *β*-carotene accumulation was based on upregulating lycopene *β*-cyclase expression (Rosati et al. 2000; Ravanello et al. 2003). Both *β-CYC* and *ε-CYC* were thought to be required for the synthesis of α-carotene and subsequently lutein in higher plants (Pogson et al. 1996). Therefore, we hypothesized that downregulating *ε-CYC* would divert the substrate, lycopene, from

Table 3 Concentrations of carotenoid compounds in seeds of *B. napus* expressing *ε-CYC* RNAi and the untransformed control DH12075 line

Plant line	β-Carotene	Lutein	Violaxanthin	Zeaxanthin	β-Cryptoxanthin	Total carotenoid	Ratio of β-carotene to lutein
DH12075	0.49 ± 0.28	3.30 ± 0.49	UD	UD	UD	5.34 ± 0.96	0.15
Construct 710-422							
BY173	15.07 ± 4.98	39.10 ± 5.33	2.43 ± 0.18	0.44 ± 0.13	0.23 ± 0.04	75.48 ± 11.23	0.39
BY228	19.52 ± 3.27	41.74 ± 3.80	1.30 ± 0.11	2.51 ± 0.69	0.08 ± 0.13	86.59 ± 8.57	0.47
BY241	4.18 ± 0.28	14.05 ± 0.21	0.61 ± 0.02	0.21 ± 0.02	0.10 ± 0.07	24.31 ± 0.56	0.30
BY269	90.76 ± 6.72	76.22 ± 3.74	1.74 ± 0.07	7.07 ± 0.13	0.30 ± 0.13	227.78 ± 2.85	1.19
BY351	6.36 ± 4.14	17.44 ± 3.46	1.09 ± 0.32	0.14 ± 0.03	UD	34.44 ± 11.22	0.36
Construct 710-423							
BY54	11.09 ± 2.06	23.15 ± 6.64	0.91 ± 0.24	0.35 ± 0.03	0.16 ± 0.06	46.95 ± 8.00	0.48
BY58	3.34 ± 1.67	9.46 ± 3.00	0.36 ± 0.13	0.33 ± 0.22	UD	19.07 ± 5.82	0.35
BY223	17.88 ± 1.25	25.29 ± 1.16	1.48 ± 0.12	0.31 ± 0.05	0.15 ± 0.01	59.81 ± 3.19	0.71
BY365	27.02 ± 4.04	37.64 ± 3.95	2.42 ± 0.25	1.73 ± 0.33	0.15 ± 0.03	94.09 ± 12.07	0.72
BY371	3.50 ± 0.31	10.36 ± 0.57	0.45 ± 0.07	0.28 ± 0.01	UD	19.15 ± 2.48	0.34

Values expressed as μg/g FW; UD, undetectable; FW, fresh weight

Each value is the mean result from triplicate ± SD

Table 4 Fatty acid content of seeds of *B. napus* expressing *ε-CYC* RNAi and the untransformed control DH12075 line

Plant line	FA content (% FW)	Palmitic acid	Steric acid	Oleic acid	Unknown 18C FA	Linoleic acid	Linolenic acid	Unknown 20C FA	Eicosanoic acid
DH12075	35.49 ± 1.08	3.58 ± 0.20	2.65 ± 0.25	62.55 ± 0.83	1.84 ± 0.17	12.33 ± 0.54	6.55 ± 0.34	1.05 ± 0.11	1.52 ± 0.07
Construct 710-422									
BY173	22.29 ± 0.36	4.60 ± 0.18	2.56 ± 0.09	52.13 ± 2.72	3.54 ± 0.41	17.58 ± 1.52	8.04 ± 0.22	0.95 ± 0.03	1.29 ± 0.04
BY228	33.02 ± 0.49	4.77 ± 0.21	3.27 ± 0.52	56.9 ± 4.70	2.22 ± 1.93	12.66 ± 2.07	5.72 ± 1.08	1.09 ± 0.11	1.14 ± 0.11
BY269	23.27 ± 2.41	4.29 ± 0.14	3.68 ± 0.11	54.15 ± 2.01	2.15 ± 1.87	14.58 ± 0.56	8.21 ± 0.52	1.21 ± 0.07	1.19 ± 0.07
BY241	24.92 ± 1.53	4.11 ± 0.07	2.57 ± 0.12	60.21 ± 2.83	2.58 ± 2.25	12.50 ± 0.30	7.41 ± 0.20	0.93 ± 0.03	1.26 ± 0.01
BY351	27.12 ± 1.67	4.27 ± 0.09	2.08 ± 0.16	62.11 ± 0.97	0.69 ± 1.20	13.27 ± 0.98	6.75 ± 0.38	0.76 ± 0.07	1.19 ± 0.09
Construct 710-423									
BY54	30.34 ± 1.91	4.24 ± 1.55	3.01 ± 0.02	52.94 ± 4.64	2.33 ± 2.03	17.32 ± 0.34	6.39 ± 0.19	1.10 ± 0.03	1.08 ± 0.02
BY223	26.14 ± 1.09	3.35 ± 1.30	2.85 ± 0.23	60.82 ± 0.66	2.80 ± 0.19	12.62 ± 1.14	7.22 ± 0.77	1.06 ± 0.20	1.27 ± 0.04
BY365	31.05 ± 1.13	4.20 ± 0.18	3.52 ± 0.20	61.22 ± 3.62	2.76 ± 2.65	10.08 ± 0.72	4.92 ± 0.56	1.27 ± 0.05	1.13 ± 0.07
BY58	35.21 ± 1.51	3.95 ± 0.12	2.36 ± 0.19	57.17 ± 0.79	1.66 ± 1.43	15.94 ± 0.42	7.85 ± 0.26	0.92 ± 0.27	1.20 ± 0.06
BY371	30.5 ± 1.04	3.85 ± 0.12	2.81 ± 0.15	63.63 ± 1.46	1.16 ± 1.02	10.05 ± 0.53	5.94 ± 0.61	1.15 ± 0.22	1.32 ± 0.08

Individual fatty acid values are the mean percentage of total fatty acids extracted ± SD

FA content is expressed as a percentage of fresh weight (FW). Results were performed in triplicate

α-carotene (and lutein) production to the other branch of the pathway leading to β-carotene. In addition, as the transcript levels of *β-CYC* were minimally affected by seed maturation, production of β-carotene should not be inhibited by lack of this enzyme. We used RNAi constructs to repress *ε-CYC* expression in *B. napus*.

As expected, reductions in *ε-CYC* transcript levels in the seeds of the transgenic plants led to increased concentrations of β-carotene compared with the untransformed wild type line, as did the concentrations of other carotenoids derived from β-carotene (Table 3). Unexpectedly, lutein concentrations were also increased dramatically in the transgenic seeds.

Our results suggest that *ε-CYC* plays a key role in the carotenoid biosynthesis pathway. Of the carotenoid biosynthesis genes examined, only transcription of *ε-CYC* and *PSY* appeared to be affected by expression of the RNAi construct (Fig. 4), yet these plants had major alterations in their carotenoid profiles. This suggests that the accumulation of carotenoids in transgenic seeds was not due to enhanced de novo biosynthesis. *ε-CYC* was reported to play a key role in controlling the ratio of lutein to *β,β*-carotenoids (Pogson et al. 1996). Control over the branch point in the pathway could be via substrate competition between *ε-CYC* and *β-CYC*. That is *ε-CYC* could be one of the rate-limiting steps in the pathway and its removal allowed the uninhibited function of *β-CYC*. The higher concentration of *β*-carotene and derivatives in the seeds of transgenic plants supports this theory.

The increase in lutein concentrations (Table 3) was unexpected as both *β*-CYC and *ε*-CYC are required for the synthesis of α-carotene and subsequently lutein in higher plants (Pogson et al. 1996). This finding appears to be inconsistent with recent reports in the literature, which showed that tuber-specific silencing of *ε-CYC* in potato resulted in significant increase in *β*-carotene (up to 14-fold), with only two transgenic lines showing minor increases in lutein levels (1.5–1.8 fold) (Diretto et al. 2006). This suggests regulation of carotenoid biosynthesis varies at the *ε*-CYC step between potato tubers and *B. napus* seeds. Three possibilities may explain the production of lutein in the *ε-CYC* silenced lines of *B. napus*: (1) The existence of additional *ε-CYC* gene copies that are not silenced by *ε-CYC* RNAi, which may have compensated for *ε*-ring formation. Southern blot analysis using enzymes with either single or no recognition sites within the cDNA sequence showed that the *B. napus* genome contains at least two homologues of *ε-CYC* (Fig. 5). These homologues need to be identified and the activity of any gene products determined in RNAi silenced and parental tissues; (2) Formation of the *ε*-ring by enzymes with a broad substrate range; enzymes with the ability to form *ε*-rings at the ends of aliphatic compounds may have led to the formation of *ε*-rings in lycopene; and (3) *ε*-CYC is not rate-limiting for lutein biosynthesis in seeds, but rather *ε-CYC* silencing may cause alteration in cellular compartments or sequestration molecules enhancing seed capacity to store lutein.

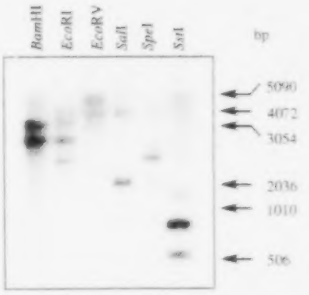


Fig. 5 Southern blot analysis of *ε-CYC* gene family in *B. napus*. Approximately 10 μg of genomic DNA was digested with *Bam*HI, *Eco*RI, *Eco*RV, *Sal*I, *Spe*I and *Sst*I restriction endonucleases. The blot was probed with a 352 bp *B. napus ε-CYC* cDNA fragment. Size markers (bp) are indicated

The increase in total carotenoid synthesis and altered ratio of lutein to *β*-carotene observed in the seeds of transgenic plants were not seen in the leaves. Differences in capacity to make and store excess carotenoids may explain the differences between the tissues. Our data showed that different tissues have different carotenoid profiles (Table 2), suggesting that regulation of biosynthesis vary from tissue to tissue. Compartmentalisation of the carotenoids may have prevented their catabolism or conversion and led to accumulation in the seeds. In seeds, carotenoids are compartmentalised to elaioplasts (lipid storing plastids), which can store a large amount of carotenoids in specialized lipoprotein-sequestering structures (Kirk and Tiliney-Bassett 1978; Bartley and Scolnik 1995; Vishnevetsky et al. 1999; Howitt and Pogson 2006). However in leaves, carotenoids are synthesized and localized with chlorophyll as a chlorophyll-carotenoid protein complex in chloroplasts (Green and Durnford 1996; Giuliano et al. 2000; Römer et al. 2002). The amounts of *β*-carotene and lutein are directly proportional to the contents of chlorophyll a and b, respectively. Therefore, carotenoid synthesis may be regulated more stringently to prevent disruption of photosynthesis (Peter and Thornber 1991; Bassi et al. 1993; Pogson et al. 1996). Another possibility is that leaf chloroplasts of transgenic plants may already have reached their limit of carotenoids and no additional storage compartments may be available for excess carotenoid molecules. Alterations in plastid morphology or

Table 5 Microarray analysis of gene expression was conducted on developing seeds of wild type DH12075 and *ε*-CYC-RNAi line BY351. Only genes with a greater than 3-fold increase in transcript level compared to the parental DH12075 line are listed

Up-regulated gene names	Expression ratio of BY351 to DH12075	Arabidopsis homolog locus name
HesB-like domain-containing protein similar to IscA (putative iron-sulfur cluster assembly protein)	13.09	At1g10500
Invertase/pectin methylesterase inhibitor family protein	7.28	At4g25260
Late embryogenesis abundant protein (M10)/LEA protein M10	5.21	At2g41280
Cytochrome b (MTCYB) (COB)	4.2	At2g07727
Chlorophyll A-B binding protein 165/180	4.02	At1g29920
Chloroplast thylakoid lumen protein	3.8	At4g02530
Armadillo/beta-catenin repeat family protein/U-box domain-containing protein	3.54	At5g62560
Protochlorophyllide reductase B	3.41	At4g27440
Pathogenesis-related thaumatin family protein	3.37	At1g19320
Meiosis protein-related (DYAD)	3.14	At5g51330
Leucine-rich repeat transmembrane protein kinase, putative	3.07	At2g31880
Expressed protein similar to myo-inositol oxygenase	3.07	At2g19800
Unknown	3.06	At2g12905

function have been observed in tissues producing excess carotenoids. A new inclusion body in plastids was observed and assumed to store excess carotenoids when a bacterial *PSY* was overexpressed in *B. napus* seeds causing a 50-fold increase in carotenoid accumulation (Shewmaker et al. 1999). In the curds of the cauliflower *Or* mutant excess *β*-carotene is stored in chromoplasts that are converted from more non-coloured plastids (Li et al. 2006). When the Or gene was introduced into potato, additional orange bodies were observed in the transgenic tubers (Li and Van Eck 2007). These suggested that enhancing the storage capacity for carotenoid accumulation is an important strategy to alter carotenoid content in plants. Further investigation is needed to determine whether alterations to plastid morphology occurred in the seeds of *ε-CYC* silenced lines.

The increase in transcript abundance (5-fold) of the late embryogenesis abundant protein M10 (LEA protein M10) in transgenic seeds (Table 5) suggests an increase in ABA production, as LEA proteins are often ABA inducible (Galau et al. 1986; Soeda et al. 2005). Greater concentrations of ABA would have the effect of fore-shortening seed maturation (Fowler and Downey 1970; Bewley 1997). This earlier maturation may be the reason why the transgenic seeds had slightly lower fatty acid contents. Fatty acid accumulation is one of the last metabolic processes to be deactivated by seed desiccation and shorter maturation times lead to lower oil concentrations (Johnson-Flanagan et al. 1992; Si and Walton 2004). Increasing ABA levels would induce earlier maturation and result in the earlier termination of storage molecule accumulation. Reduction in oleic acid concentration in transgenic seeds may be related to a reduction in the expression of stearoyl-ACP desaturase (Table 5), which inserts a *cis* double bond at the 9 position of C18:0-ACP (Ohlrogge and Jaworski 1997). Another possible reason for a decreased fatty acid could be the diversion of more of the carbon pool to carotenoid biosynthesis, which depleted available carbon precursor for fatty acid biosynthesis. Determination of ABA content and examination of the lipid:protein ratio in the seeds

from the RNAi lines would help determine if reducing *ε-CYC* expression, or increasing carotenoid content, affects the biosynthesis and accumulation of ABA and fatty acids in the seeds.

Acknowledgements We are grateful to Mr. Delwin Epp for technical assistance with *B. napus* tissue culture and Dr. Branimir Gjetvaj for assistance with the microarray analysis. We thank Drs. Kevin Falk, Kevin Rozwadowski and Bhinu V.S. for critical reading of the manuscript, and for helpful suggestions. Funding for this project was provided by the Saskatchewan Agriculture Development Fund.

References

Bartley GE, Scolnik PA (1995) Plant carotenoid: pigments for photoprotection, visual attraction, and human health. Plant Cell 7:1027–1038

Bassi R, Pineau B, Dainese P, Marquardt J (1993) Carotenoid-binding proteins of photosystem II. Eur J Biochem 212:297–303

Bewley JD (1997) Seed germination and dormancy. Plant Cell 9:1055–1066

Botella-Pavía P, Rodríguez-Concepción M (2006) Carotenoid biotechnology in plants for nutritionally improved foods. Physiol Plant 126:369–381

Carpenter CD, Simon AE (1998) Preparation of RNA. In: Martinez-Zapater JM, Salinas J (eds) Methods in molecular biology, vol. 82. Arabidopsis protocols. Humana Press, Totowa, NJ, pp 85–89

Church G, Gilbert W (1984) Genome sequencing. Proc Natl Acad Sci USA 81:1991–1995

Cunningham FX Jr, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 49:557–583

Cunningham FX Jr, Gantt E (2001) One ring or two? Determination of ring number in carotenoids by lycopene ε-cyclases. Proc Natl Acad Sci USA 98:2905–2910

Cuttriss AJ, Pogson BJ (2004) Carotenoids. In: Davies KM (ed) Plant pigments and their manipulation. CRC Press, Boca Raton, FL, pp 57–91

Demmig-Adams B, Gilmore AM, Adams WW III (1996) In vivo functions of carotenoids in higher plants. FASEB J 10:403–412

Demmig-Adams B, Adams WW III (2002) Antioxidants in photosynthesis and human nutrition. Science 298:2149–2153

Diretto G, Tavazza R, Welsch R, Pizzichini D, Mourgues F, Papacchioli V, Beyer P, Giuliano G (2006) Metabolic engineering of potato tuber carotenoids through tuber-specific silencing of lycopene epsilon cyclase. BMC Plant Biol 6:13

Diretto G, Welsch R, Tavazza R, Mourgues F, Pizzichini D, Beyer P, Giuliano G (2007) Silencing of beta-carotene hydroxylase increases total carotenoid and beta-carotene levels in potato tubers. BMC Plant Biol 7:11

Ducreux LJM, Morris WL, Hedley PE, Shepherd T, Davies HV, Millam S, Taylor MA (2005) Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. J Exp Bot 56:81–89

Fowler DB, Downey RK (1970) Lipid and morphological changes in developing rapeseed, *Brassica napus*. Can J Plant Sci 50:233–247

Fraser PD, Bramley PM (2004) The biosynthesis and nutritional uses of carotenoids. Prog Lipid Res 43:228–265

Galau GA, Hughes DW, Dure L (1986) Abscisic-acid induction of cloned cotton late embryogenesis-abundant (lea) messenger-RNAs. Plant Mol Biol 7:155–170

Giuliano G, Bartley GE, Scolnik PA (1993) Regulation of carotenoid biosynthesis during tomato development. Plant Cell 5:379–387

Giuliano G, Aquilani R, Dharmapuri S (2000) Metabolic engineering of plant carotenoids. Trends Plant Sci 5:406–409

Goodwin TW (1980) The biochemistry of the carotenoids, 2nd edn., vol 1. Chapman & Hall, London, pp 377

Green BR, Durnford DG (1996) The chlorophyll-carotenoid proteins of oxygenic photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 47:685–714

Howitt CA, Pogson BJ (2006) Carotenoid accumulation and function in seeds and non-green tissues. Plant Cell Environ 29:435–445

Johnson-Flanagan AM, Huirwen Z, Geng X-M, Brown DCW, Nykiforuk CL, Singh S (1992) Frost, abscisic acid, and desiccation hasten embryo development in *Brassica napus*. Plant Physiol 99:700–706

Kirk JT, Tilney-Bassett RA (1978) Proplastids, etioplasts, amyloplasts, chromoplasts and other plastids. In: Kirck ST, Tilney-Bassett RA (eds) The plastids:their chemistry, structure, growth and inheritance. Elsevier/North Holland, Biomedical Press, Amsterdam, pp 217–239

Krinsky NI, Johnson EJ (2005) Carotenoid actions and their relation to health and disease. Mol Aspects Med 26:459–516

Kuhlbrandt W, Wang DN, Fujiyoshi Y (1994) Atomic model of plant light-harvesting complex by electron crystallography. Nature 367:614–621

Landrum JT, Bone RA (2004) Dietary lutein and zeaxanthin: reducing the risk of macular degeneration. Agro Food Industry Hi-Tech 15:22–25

Lakshman MR, Okoh C (1993) Enzymatic conversion of all trans-beta-carotene to retinal. Meth Enzymol 214:256–269

Li L, Lu S, Cosman KM, Earle ED, Garvin DF, O'Neill J (2006) β-Carotene accumulation induced by cauliflower *Or* gene is not due to an increase capacity of biosynthesis. Phytochemistry 67:1177–1184

Li L, Van Eck J (2007) Metabolic engineering of carotenoid accumulation by creating a metabolic sink. Transgenic Res DOI 10.1007/s11248-007-9111-1

Lindgren L, Stahlberg KG, Hoglund AS (2003) Seed-specific overexpression of an endogenous *Arabidopsis* phytoene synthase gene results in delayed germination and increased levels of carotenoids, chlorophyll, and abscisic acid. Plant Physiol 132:779–785

Mayne ST (1996) Beta-carotene, carotenoids and disease prevention in humans. FASEB J 10:690–701

Moloney MM, Walker JM, Sharma KK (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep 8:238–242

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56:165–185

Ohlrogge JB, Jaworski JG (1997) Regulation of fatty acid synthesis. Annu Rev Plant Physiol Plant Mol Biol 48:109–136

Peter GF, Thornber JP (1991) Biochemical composition and organization of higher plant photosystem II light-harvesting pigment-protein. J Biol Chem 266: 16745–16754

Pogson BJ, McDonald K, Truong M, Britton G, DellaPenna D (1996) Arabidopsis carotenoid mutants demonstrate lutein is not essential for photosynthesis in higher plants. Plant Cell 8:1627–1639

Ravanello MP, Ke D, Alvarez J, Huang B, Shewmaker CK (2003) Coordinate expression of multiple bacterial carotenoid genes in canola leading to altered carotenoid production. Metab Eng 5:255–263

Rock CD, Zeevaart JAD (1991) The aba mutant of Arabidopsis thaliana is impaired in epoxy-carotenoid biosynthesis. Proc Natl Acad Sci USA 88:7496–7499

Römer S, Lübeck J, Kauder F, Steiger S, Adomat C, Sandmann G (2002) Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. Metab Eng 4:263–272

Rosati C, Aquilani R, Dharmapuri S, Pallara P, Marusic C, Tavazza R, Bouvier F, Camara B, Giuliano G (2000) Metabolic engineering of beta-carotene and lycopene content in tomato fruit. Plant J 24:413–419

Shewmaker CK, Sheey JA, Daley M, Colburn S, Ke DY (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. Plant J 20:401–412

Si P, Walton GH (2004) Determinants of oil concentration and seed yields in canola and Indian Mustard in the lower rainfall areas of Western Australia. Aust J Agric Res 55:367–377

Soeda Y, Konings MCJM, Vorst O, van Houwelingen AMML, Stoopen GM, Maliepaard CA, Kodde J, Bino RJ, Groot SPC, van der Geest AHM (2005) Gene expression programs during *Brassica oleracea* seed maturation, osmopriming, and germination are indicators of progression of the germination process and the stress tolerance level. Plant Physiol 137:354–368

Stickforth P, Steiger S, Hess WR, Sandmann G (2003) A novel type of lycopene ε-cyclase in the marine *cyanobacterium Prochlorococcus* marinus MED4. Arch Microbiol 179:409–415

Taylor M, Ramsay G (2005) Carotenoid biosynthesis in plant storage organs: recent advances and prospects for improving plant food quality. Physiol Plant 124:143–151

Vishnevetsky M, Ovadis M, Vainstein A (1999) Carotenoid sequestration in plants: the role of carotenoid-associated proteins. Trends Plant Sci 4:232–235

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid –free) rice endosperm. Science 287:303–305

Young AJ (1993) Factors that affect the carotenoid composition of higher plants and algae. In: Young AJ, Britton G (eds) Carotenoids in photosynthesis. Chapman and Hall, London, pp 161–205

Young LW, Jalink H, Denkert R, Reaney MTJ (2006) Factors affecting the density of *Brassica napus* seeds. Seed Sci & Technol 34:633–645

Agriculture and Agri-Food Canada / Agriculture et Agroalimentaire Canada

Molecular and Functional Characterization of ß-Carotene Hydroxylase of *Adonis aestivalis*

Bianyun Yu, Ulrike Schäfer, Derek Lydiate, and Abdelali Hannoufa

Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, SK, S7N 0X2, Email: yub@agr.gc.ca

Introduction

Carotenoids, including xanthophylls and carotenes, are ubiquitous secondary metabolites derived from the isoprenoid pathway. The importance of these metabolites is evident from their use as: 1) additives in animal, poultry and fish feed formulations; 2) precursors of vitamins important in human and animal nutrition; 3) colourants in the food and cosmetics industries; 4) antioxidants with potential anti-cancer therapeutic properties. Plants species belonging to the genus *Adonis* are known to accumulate high levels of carotenoids, such as zeaxanthin and lutein. To further our understanding of the biosynthetic pathway of carotenoids in higher plants, we cloned a cDNA from the flower petals of *Adonis aestivalis*. This cDNA encodes a novel ß-carotene hydroxylase, CrtH, which catalyzes the conversion of ß-carotene to zeaxanthin.

Results and Discussion

Cloning of ß-Carotene Hydroxylase of *Adonis aestivalis*

Degenerate primers were designed based on the conserved domains of ß-carotene hydroxylases of various organisms (**Fig. 1**). Using these primers, a 363bp fragment was amplified by RT-PCR using RNA from the flower petals of *Adonis aestivalis*. The 363bp fragment was then used for the cloning of the 3'- and 5'-ends using respective 3'- and 5'- RACE techniques (Rapid Amplification cDNA Ends) (**Fig. 2**). The isolated full-length cDNA clone of ß-carotene hydroxylase (*CrtH*) has 1.187 kbp and a total transcript size of ~1.35 kb.

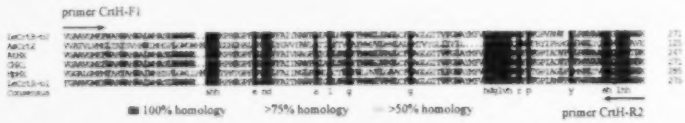


Figure 1. Alignment of amino acid sequences of ß-carotene hydroxylases from various organisms, and positions of the degenerate primers, CrtH-F1 and CrtH-R2, used to amplify the conserved 363bp fragment. *Lycopersicon esculentum LeCrtR-b1*(Y14809) and *LeCrtR-b2* (Y14810) [Hirschberg,1998 *in* Britton, G., Liaaen-Jensen, S. and Pfander, H. (*Eds.*), *Carotenoids: Biosynthesis and Metabolism*, Vol 3, pp 149-194, Birkhaeuser Verlag, Berlin]; *Alcaligenes sp. AsCrtZ* (D58422) [Misawa, *et al.* 1995, *J. Bacteriol.* 177: 6575]; *Arabidopsis thaliana AtHX* (U58919) [Sun *et al.* 1996, *J. Biol. Chem.* 271: 24349]; *Citrus unshiu CHX1*(AF296158) [Kim *et al.* 2001, *Plant Sci.* 161: 1005]; *Haematococcus pluvialis HpHX* (AF162276) [Linden, 1999, *Biochim. Biophys. Acta* 1446: 203].

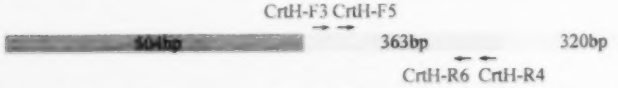


Figure 2. Rapid amplification cDNA ends of ß-carotene hydroxylase. Primers CrtH-F3 and CrtH-F5 were used for isolation of the 3'-end (320bp), and primers CrtH-R4 and CrtH-R6 for the 5'-end (504bp) of *CrtH*.

Acknowledgement

Funding for this project is provided by the Saskatchewan Agriculture Development Fund.

Sequence Analysis and Southern Blot

The *CrtH* cDNA encodes a predicted protein of 309 amino acids with a molecular weight of ~35 kDa and a pI of 9.15. An alignment of the deduced protein sequence of CrtH with those of ß-carotene hydroxylases from other organisms showed that they shared high level of similarity, including ~70% amino acid identity with plant ß-carotene hydroxylases (**Fig. 3**). The chloroplast transit peptide prediction software Chloroplast v1.1 (http://www.cbs.dtu.dk/services/ChloroP/) predicted a chloroplast transit peptide cleavage site between Val[59] and Ala[60], consistent with the presumed chloroplastic location of a carotenoid biosynthetic enzyme. Four transmembrane helical regions were predicted. Four conservatively spaced histidine motifs were also present, HXXXXH and HXXH (**Fig. 4**). These motifs are characteristic of membrane fatty acid desaturases and membrane hydrocarbon hydroxylases. Southern blot analysis showed that ß-carotene hydroxylase *CrtH* was encoded by a multi-copy gene family of at least three members in *Adonis aestivalis* (**Fig. 5**). Northern blot analysis of the *CrtH* transcript in different organs of *Adonis aestivalis* revealed that it was highly expressed in flower petals, roots, and stems, with relatively much lower expression in leaves and developing seeds (**Fig. 6**).

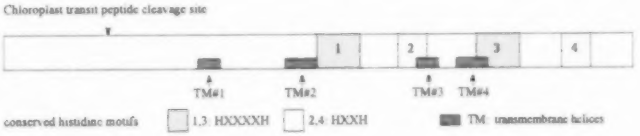


Figure 4. Conserved histidine motifs and transmembrane helices of CrtH

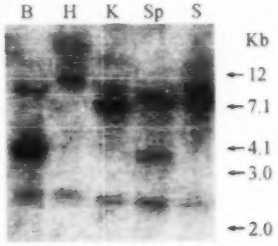


Figure 5. Southern blot analysis of ß-carotene hydroxylase (*CrtH*) of *Adonis aestivalis*. About 10µg of genomic DNA was digested with *BamHI* (B), *HindIII* (H), *KpnI* (K), *SpeI* (SP), and *SstI* (S) restriction endonucleases, which have no recognition sites in the cDNA sequence of *CrtH*, separated on a 0.8% agarose gel, transferred onto Hybond-XL membrane and hybridized with 201bp *CrtH* -specific fragment from the 3'-end labeled with [^{32}P] dCTP.

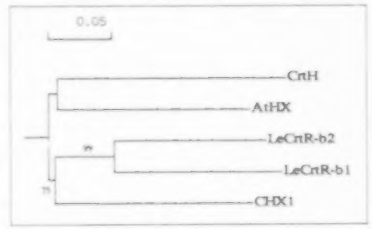


Figure 3. Phylogenetic relationship between the ß-carotene hydroxylase of *Adonis aestivalis* (*CrtH*) and those of other organisms. *Lycopersicon esculentum LeCrtR-b1*(Y14809) and *LeCrtR-b2* (Y14810), *Alcaligenes sp. AsCrtZ* (D58422), *Arabidopsis thaliana AtHX* (U58919), *Citrus unshiu CHX1*(AF296158).

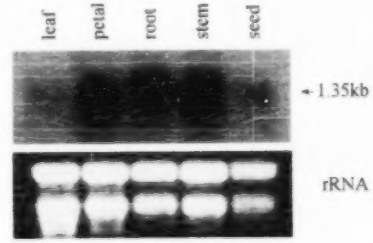


Figure 6. Expression profile of ß-carotene hydroxylases (*CrtH*) in different organs of *Adonis aestivalis*. Approximately 15µg of total RNA was electrophoresed on 1.2% agarose gel containing 6.3% formaldehyde, and blotted onto Hybond-XL membrane. Probe was the same as that used for Southern analysis.

Conclusion

We have cloned the full-length cDNA sequence of ß-carotene hydroxylase from *Adonis aestivalis*. Features of the translated protein are consistent with a plastid-localized ß-carotene hydroxylase. These features include a plastid transit peptide, transmembrane helices and conserved histidine motifs. Further biochemical and molecular characterization of this protein is currently underway. This gene is an additional tool that will allow for further understanding, dissection, and modification of the biosynthetic pathway of carotenoids in higher plants.

Canada

Molecular and Functional Characterization of ß-Carotene Hydroxylase of *Adonis aestivalis*

Bianyun Yu, Ulrike Schäfer, Derek Lydiate, and Abdelali Hannoufa

Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, SK, S7N 0X2. Email: yub@agr.gc.ca

Introduction

Carotenoids, including xanthophylls and carotenes, are ubiquitous secondary metabolites derived from the isoprenoid pathway. The importance of these metabolites is evident from their use as: 1) additives in animal, poultry and fish feed formulations; 2) precursors of vitamins important in human and animal nutrition; 3) colourants in the food and cosmetics industries; 4) antioxidants with potential anti-cancer therapeutic properties. Plants species belonging to the genus *Adonis* are known to accumulate high levels of carotenoids, such as zeaxanthin and lutein. To further our understanding of the biosynthetic pathway of carotenoids in higher plants, we cloned a cDNA from the flower petals of *Adonis aestivalis*. This cDNA encodes a novel ß-carotene hydroxylase, CrtH, which catalyzes the conversion of ß-carotene to zeaxanthin.

Results and Discussion

Cloning of ß-Carotene Hydroxylase of *Adonis aestivalis*

Degenerate primers were designed based on the conserved domains of ß-carotene hydroxylases of various organisms (**Fig. 1**). Using these primers, a 363bp fragment was amplified by RT-PCR using RNA from the flower petals of *Adonis aestivalis*. The 363bp fragment was then used for the cloning of the 3'- and 5'-ends using respective 3'- and 5'-RACE techniques (Rapid Amplification cDNA Ends) (**Fig. 2**). The isolated full-length cDNA clone of ß-carotene hydroxylase (*CrtH*) has 1.187 kbp and a total transcript size of ~1.35 kb.

Figure 1. Alignment of amino acid sequences of ß-carotene hydroxylases from various organisms, and positions of the degenerate primers, CrtH-F1 and CrtH-R2, used to amplify the conserved 363bp fragment. *Lycopersicon esculentum LeCrtR-b1* (Y14809) and *LeCrtR-b2* (Y14810) [Hirschberg 1998 *in* Britton, G., Liaaen-Jensen, S. and Pfander, H. (*Eds.*) *Carotenoids: Biosynthesis and Metabolism*, Vol 3, pp 149-194, Birkhaeuser Verlag, Berlin]; *Alcaligenes sp. AsCrtZ* (D58422) [Misawa, *et al.* 1995, *J. Bacteriol.* 177: 6575]; *Arabidopsis thaliana AtHX* (U58919) [Sun *et al.* 1996, *J. Biol. Chem.* 271: 24349]; *Citrus unshiu CHX1* (AF296158) [Kim *et al.* 2001, *Plant Sci.* 161: 1005]; *Haematococcus pluvialis HpHX* (AF162276) [Linden, 1999, *Biochim. Biophys. Acta* 1446: 203].

Figure 2. Rapid amplification cDNA ends of ß-carotene hydroxylase. Primers CrtH-F3 and CrtH-F5 were used for isolation of the 3'-end (320bp), and primers CrtH-R4 and CrtH-R6 for the 5'-end (504bp) of *CrtH*.

Acknowledgement

Funding for this project is provided by the Saskatchewan Agriculture Development Fund.

Sequence Analysis and Southern Blot

The *CrtH* cDNA encodes a predicted protein of 309 amino acids with a molecular weight of ~35 kDa and a pI of 9.15. An alignment of the deduced protein sequence of CrtH with those of ß-carotene hydroxylases from other organisms showed that they shared high level of similarity, including ~70% amino acid identity with plant ß-carotene hydroxylases (**Fig. 3**). The chloroplast transit peptide prediction software ChloroplastV1.1 (http://www.cbs.dtu.dk/services/ChloroP/) predicted a chloroplast transit peptide cleavage site between Val[illegible] and Ala[illegible], consistent with the presumed chloroplastic location of a carotenoid biosynthetic enzyme. Four transmembrane helical regions were predicted. Four conservatively spaced histidine motifs were also present: HXXXXH and HXXH (**Fig. 4**). These motifs are characteristic of membrane fatty acid desaturases and membrane hydrocarbon hydroxylases. Southern blot analysis showed that ß-carotene hydroxylase *CrtH* was encoded by a multi-copy gene family of at least three members in *Adonis aestivalis* (**Fig. 5**). Northern blot analysis of the *CrtH* transcript in different organs of *Adonis aestivalis* revealed that it was highly expressed in flower petals, roots, and stems, with relatively much lower expression in leaves and developing seeds (**Fig. 6**).

Figure 3. Phylogenetic relationship between the ß-carotene hydroxylase of *Adonis aestivalis* (*CrtH*) and those of other organisms. *Lycopersicon esculentum LeCrtR-b1* (Y14809) and *LeCrtR-b2* (Y14810), *Alcaligenes sp. AsCrtZ* (D58422), *Arabidopsis thaliana AtHX* (U58919), *Citrus unshiu CHX1* (AF296158).

Figure 4. Conserved histidine motifs and transmembrane helices of CrtH.

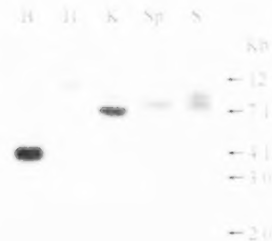


Figure 5. Southern blot analysis of ß-carotene hydroxylase (*CrtH*) of *Adonis aestivalis*. About 10µg of genomic DNA was digested with *BamHI* (B), *HindIII* (H), *KpnI* (K), *SpeI* (SP), and *SalI* (S) restriction endonucleases, which have no recognition sites in the cDNA sequence of *CrtH*, separated on a 0.8% agarose gel, transferred onto Hybond-XL membrane and hybridized with 201bp *CrtH*-specific fragment from the 3'-end labeled with [^{32}P] dCTP.

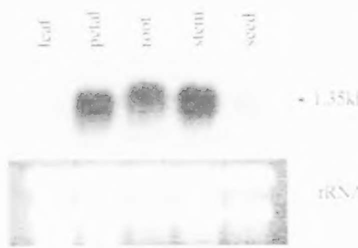


Figure 6. Expression profile of ß-carotene hydroxylases (*CrtH*) in different organs of *Adonis aestivalis*. Approximately 15µg of total RNA was electrophoresed on 1.2% agarose gel containing 6.3% formaldehyde, and blotted onto Hybond-XL membrane. Probe was the same as that used for Southern analysis.

Conclusion

We have cloned the full-length cDNA sequence of ß-carotene hydroxylase from *Adonis aestivalis*. Features of the translated protein are consistent with a plastid-localized ß-carotene hydroxylase. These features include a plastid transit peptide, transmembrane helices and conserved histidine motifs. Further biochemical and molecular characterization of this protein is currently underway. This gene is an additional tool that will allow for further understanding, dissection, and modification of the biosynthetic pathway of carotenoids in higher plants.

Canada

Molecular Cloning and Characterization of ß-Carotene Hydroxylase and ß-Carotene Ketolase from *Adonis aestivalis*

Bianyun Yu, Derek Lydiate, Ulrike Schäfer, Gordon Gropp, and Abdelali Hannoufa

Molecular Genetics Section, Agriculture & Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, SK, Canada S7N 0X2, email: hannoufaa@agr.gc.ca

Introduction

Xanthophylls are important nutrients, and are essential supplements in feed formulations. Flower petals of the plant *Adonis aestivalis* are known for their propensity to accumulate significant amounts of xanthophylls, including astaxanthin. To gain insight in the biosynthesis of xanthophylls, we set out to clone *Adonis aestivalis* genes encoding ß-carotene hydroxylase (*CrtH*) and ß-carotene ketolase (*CrtK*), which together convert ß-carotene to astaxanthin (**Figure 1**).

Results

Degenerate primers were designed based on the conserved domains of ß-carotene hydroxylases and ß-carotene ketolases of various organisms (**Figures 2a and 2b**). Using these primers, partial sequences of the two genes were isolated by RT-PCR. Isolation of the full length cDNA clones was attempted by employing 5' and 3' RACE (Rapid Amplification cDNA Ends) on the partial sequences (**Figures 3a** and **3b**). A full length ß-carotene hydroxylase cDNA of 1187bp was isolated using RACE, and showed high homology with ß-carotene hydroxylases from other higher plants (**Figure 4**).

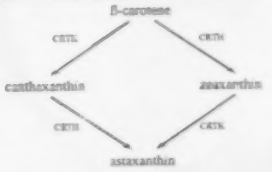


Figure 1. Astaxanthin biosynthesis from ß-carotene. ß-carotene hydroxylase, CRTH; ß-carotene ketolase, CRTK.

Cloning of ß-carotene hydroxylase and ß-carotene ketolase of *Adonis aestivalis*

Using RT-PCR and degenerate primers specific to the conserved domains of ß-carotene hydroxylases (**Figure 2a**) and ß-carotene ketolases (**Figure 2b**), a 363bp fragment of ß-carotene hydroxylase and a 369bp fragment of ß-carotene ketolase were amplified from *Adonis aestivalis*.

Figure 2a. Alignment of amino acid sequences of ß-carotene hydroxylases from various organisms. *LeCrtR-b1*(Y14809) and *LeCrtR-b2* (Y14810) are from *Lycopersicon esculentum*, *AsCrtZ* (D58422) is from *Alcaligenes sp.*, *AtHX* (U58919) is from *Arabidopsis thaliana*, *CHX1*(AF296158) is from *Citrus unshiu*, *HpHX* (AF162276) is from *Haematococcus pluvialis*. Degenerate primers, CrtH-F1 and CrtH-R2, specific to the conserved domain were designed for use with RT-PCR.

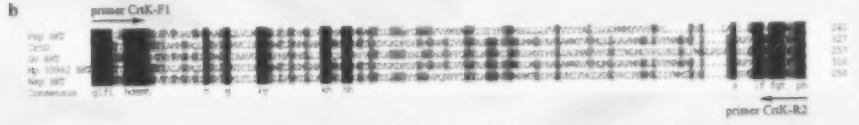


Figure 2b. Alignment of amino acid sequences of ß-carotene ketolases from various organisms *Psp BKT* (D58420) is from *Paracoccus sp.* MBIC1143, *CrtO* (CAA60478) is from *Haematococcus pluvialis*, *GvBKT*(BAC89669) is from *Gloeobacter violaceus*, *Hp39982 BKT* (Q39982) is from *Haematococcus pluvialis*, *Nsp BKT* (NP_487229) is from *Nostoc sp.* PCC 7120. Degenerate primers, CrtK-F1 and CrtK-R2, specific to the conserved domain were designed for RT-PCR.

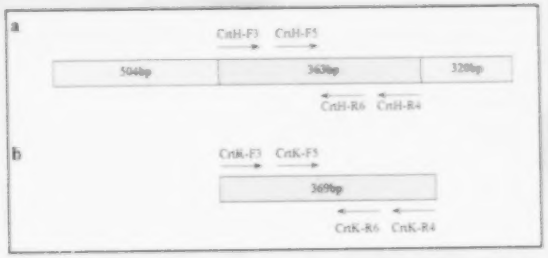


Figure 3. Rapid amplification cDNA ends of ß-carotene hydroxylase and ß-carotene ketolase. Primer CrtH-F3 and CrtH-F5 were used for isolation of hydroxylase 3'end, CrtH-R4 and CrtH-R6 for hydroxylase 5'end (**3a**). 504bp of 5' end and 320bp of 3' end of hydroxylase were cloned. CrtK-F3 and CrtK-F5 are being used for isolation of ketolase 3' end, CrtK-R4 and CrtK-R6 for ketolase 5'end (**3b**).

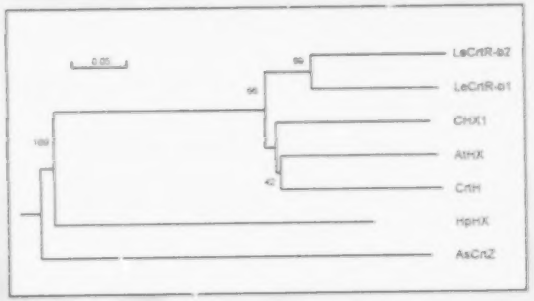


Figure 4. Phylogenetic relationship between the ß-carotene hydroxylase of *Adonis aestivalis* (*CrtH*) and those of other organisms. Organisms of other genes are as indicated in **Figure 2a**.

Expression patterns of ß-carotene hydroxylase and ß-carotene ketolase

Northern blot analysis was conducted to determine the expression patterns of the two genes in *Adonis aestivalis*. As shown in **Figure 5**, both genes are highly expressed in flower petals, roots, and stems with lower or no expression in leaves. The level of expression of ß-carotene ketolase in different organs appears to be much lower than that of ß-carotene hydoxylase.

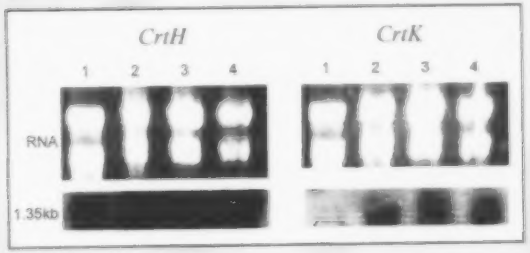


Figure 5. Expression profiles of ß-carotene hydroxylase (*CrtH*) and ß-carotene ketolase (*CrtK*) in different organs of *Adonis aestivalis*. **1** leaf, **2** petal, **3** root, **4** stem

Conclusion

We have cloned full length and partial cDNA sequences of *Adonis aestivalis* ß-carotene hydroxylase and ß-carotene ketolase, respectively. These genes will be useful tools in understanding and modifying the biosynthesis of xanthophylls in higher plants.

Acknowledgment

Funding for this project is provided by the Saskatchewan Agriculture Development Fund.

Canada

Agriculture and Agri-Food Canada | Agriculture et Agroalimentaire Canada

Molecular Cloning and Characterization of ß-Carotene Hydroxylase and ß-Carotene Ketolase from *Adonis aestivalis*

Bianyun Yu, Derek Lydiate, Ulrike Schäfer, Gordon Gropp, and Abdelali Hannoufa

Molecular Genetics Section, Agriculture & Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, SK, Canada S7N 0X2, email: hannoufaa@agr.gc.ca

Introduction

Xanthophylls are important nutrients, and are essential supplements in feed formulations. Flower petals of the plant *Adonis aestivalis* are known for their propensity to accumulate significant amounts of xanthophylls, including astaxanthin. To gain insight in the biosynthesis of xanthophylls, we set out to clone *Adonis aestivalis* genes encoding ß-carotene hydroxylase (*CrtH*) and ß-carotene ketolase (*CrtK*), which together convert ß-carotene to astaxanthin (**Figure 1**).

Results

Degenerate primers were designed based on the conserved domains of ß-carotene hydroxylases and ß-carotene ketolases of various organisms (**Figures 2a** and **2b**). Using these primers, partial sequences of the two genes were isolated by RT-PCR. Isolation of the full length cDNA clones was attempted by employing 5' and 3' RACE (Rapid Amplification cDNA Ends) on the partial sequences (**Figures 3a** and **3b**). A full length ß-carotene hydroxylase cDNA of 1187bp was isolated using RACE, and showed high homology with ß-carotene hydroxylases from other higher plants (**Figure 4**).

Figure 1. Astaxanthin biosynthesis from ß-carotene. ß-carotene hydroxylase, CRTH; ß-carotene ketolase, CRTK.

Cloning of ß-carotene hydroxylase and ß-carotene ketolase of *Adonis aestivalis*

Using RT-PCR and degenerate primers specific to the conserved domains of ß-carotene hydroxylases (**Figure 2a**) and ß-carotene ketolases (**Figure 2b**), a 363bp fragment of ß-carotene hydroxylase and a 369bp fragment of ß-carotene ketolase were amplified from *Adonis aestivalis*.

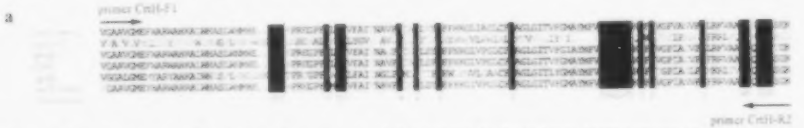


Figure 2a. Alignment of amino acid sequences of ß-carotene hydroxylases from various organisms. *LeCrtR-b1*(Y14809) and *LeCrtR-b2* (Y14810) are from *Lycopersicon esculentum*. *AsCrtZ* (D58422) is from *Alcaligenes sp.*; *AtHX* (U58919) is from *Arabidopsis thaliana*; *CHX1*(AF296158) is from *Citrus unshiu*; *HpHX* (AF162276) is from *Haematococcus pluvialis*. Degenerate primers, CrtH-F1 and CrtH-R2, specific to the conserved domain were designed for use with RT-PCR.

Figure 2b. Alignment of amino acid sequences of ß-carotene ketolases from various organisms. *Psp BKT* (D58420) is from *Paracoccus sp.* MBIC1143; *CrtO* (CAA60478) is from *Haematococcus pluvialis*; *GvBKT*(BAC89669) is from *Gloeobacter violaceus*; *Hp39982 BKT* (Q39982) is from *Haematococcus pluvialis*; *Nsp BKT* (NP_487229) is from *Nostoc sp.* PCC 7120. Degenerate primers, CrtK-F1 and CrtK-R2, specific to the conserved domain were designed for RT-PCR.

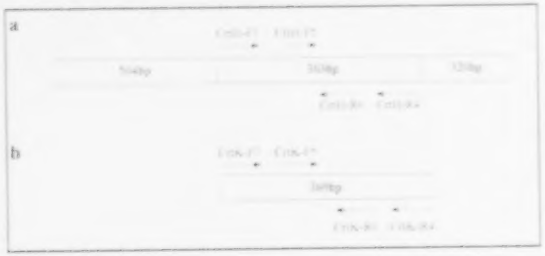


Figure 3. Rapid amplification cDNA ends of ß-carotene hydroxylase and ß-carotene ketolase. Primer CrtH-F3 and CrtH-F5 were used for isolation of hydroxylase 3'end. CrtH-R4 and CrtH-R6 for hydroxylase 5'end (**3a**) 504bp of 5' end and 320bp of 3' end of hydroxylase were cloned. CrtK-F3 and CrtK-F5 are being used for isolation of ketolase 3' end. CrtK-R4 and CrtK-R6 for ketolase 5'end (**3b**).

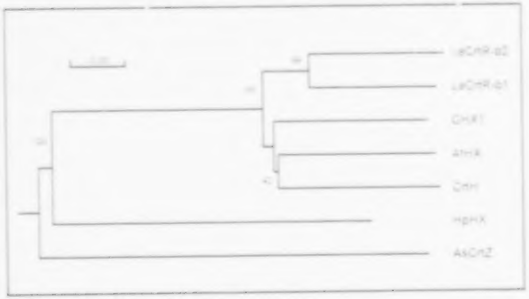


Figure 4. Phylogenetic relationship between the ß-carotene hydroxylase of *Adonis aestivalis* (*CrtH*) and those of other organisms. Organisms of other genes are as indicated in **Figure 2a**.

Expression patterns of ß-carotene hydroxylase and ß-carotene ketolase

Northern blot analysis was conducted to determine the expression patterns of the two genes in *Adonis aestivalis*. As shown in **Figure 5**, both genes are highly expressed in flower petals, roots, and stems with lower or no expression in leaves. The level of expression of ß-carotene ketolase in different organs appears to be much lower than that of ß-carotene hydoxylase.

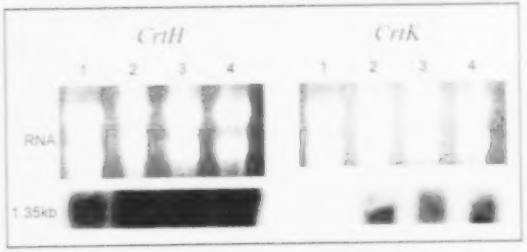


Figure 5. Expression profiles of ß-carotene hydroxylase (*CrtH*) and ß-carotene ketolase (*CrtK*) in different organs of *Adonis aestivalis*. **1** leaf, **2** petal, **3** root, **4** stem.

Conclusion

We have cloned full length and partial cDNA sequences of *Adonis aestivalis* ß-carotene hydroxylase and ß-carotene ketolase, respectively. These genes will be useful tools in understanding and modifying the biosynthesis of xanthophylls in higher plants.

Acknowledgment

Funding for this project is provided by the Saskatchewan Agriculture Development Fund.

Canada

Molecular and Functional Characterization of ß-Carotene Hydroxylase of *Adonis aestivalis*

Bianyun Yu, Ulrike Schäfer, Derek Lydiate, and Abdelali Hannoufa

Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, SK, S7N 0X2, Email: yub@agr.gc.ca

Introduction

Carotenoids, including xanthophylls and carotenes, are ubiquitous secondary metabolites derived from the isoprenoid pathway. The importance of these metabolites is evident from their use as: 1) additives in animal, poultry and fish feed formulations; 2) precursors of vitamins important in human and animal nutrition; 3) colourants in the food and cosmetics industries; 4) antioxidants with potential anti-cancer therapeutic properties. Plant species belonging to the genus *Adonis* are known to accumulate high levels of carotenoids, such as zeaxanthin and lutein. To further our understanding of the biosynthetic pathway of carotenoids in higher plants, we cloned a cDNA from the flower petals of *Adonis aestivalis*. This cDNA encodes a novel ß-carotene hydroxylase, CrtH, which catalyzed the conversion of ß-carotene to zeaxanthin via ß-cryptoxanthin in *E. coli* functional assays. Expression of *CrtH* in a double knockout mutant of *Arabidopsis thaliana* having T-DNA inserts in the two loci encoding ß-carotene hydroxylase enhanced the biosynthesis of zeaxanthin and violaxanthin.

Results and Discussion

CrtH encodes a novel ß-Carotene Hydroxylase in *Adonis aestivalis*

A cDNA clone, *CrtH*, was isolated from the flower petals of *Adonis aestivalis* using PCR-based methods. The isolated full-length cDNA clone of *CrtH* has 1187 bp and a total transcript size of ~1.35 kb. The *CrtH* cDNA encodes a predicted protein of 309 amino acids with a molecular weight of ~35 kDa and a pI of 9.15. An alignment of the deduced protein sequence of CrtH with those of ß-carotene hydroxylases from other organisms showed that they shared high level of similarity, including ~70% amino acid identity with plant ß-carotene hydroxylases. The chloroplast transit peptide prediction software Chloroplast v1.1 (http://www.cbs.dtu.dk/services/ChloroP/) predicted a chloroplast transit peptide cleavage site between Val^{58} and Ala^{59}, consistent with the presumed chloroplastic location of a carotenoid biosynthetic enzyme. Four transmembrane helical regions were predicted. Four conservatively spaced histidine motifs were also present, HXXXXH and HXXH (**Fig. 1**). These motifs are characteristic of membrane fatty acid desaturases and membrane hydrocarbon hydroxylases.

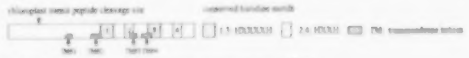


Fig. 1 Conserved histidine motifs and transmembrane helices of CrtH

Southern and Northern Blot Analysis of *CrtH*

Southern blot analysis showed that ß-carotene hydroxylase *CrtH* was encoded by a multi-copy gene family of at least three members in *Adonis aestivalis* (**Fig. 2**). Northern blot analysis of the *CrtH* transcript in different organs of *Adonis aestivalis* revealed that it was highly expressed in flower petals, root, and stems, with relatively much lower expression in leaves and developing seeds (**Fig. 3**).

Acknowledgement

We thank Dr. Norihiko Misawa (Kirin Brewery Co. Ltd, Kanagawa, Japan) for the kind gift of plasmid pACCAR16ΔcrtX and Dr. Dean DellaPenna (Michigan State University, Michigan, USA) for generously providing the seeds of *Arabidopsis thaliana* b1b2 double knockout mutant. Funding for this project is provided by the Saskatchewan Agriculture Development Fund.

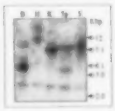


Fig. 2 Southern blot analysis of *CrtH*. About 10µg of genomic DNA was digested with *BamHI* (B), *HindIII* (H), *KpnI* (K), *SpeI* (SP), and *SstI* (S) restriction endonucleases, which have no recognition sites in the cDNA sequence of *CrtH*. The blot was probed with 201bp of *CrtH* specific fragment labeled with [α-^{32}P]dCTP.

Fig. 3 Expression profile of *CrtH* in different organs of *Adonis aestivalis*. The probe was the same as that used for Southern analysis.

Functional Assay of *CrtH* in *E. coli*

A functional assay was conducted in *E. coli* JM101, in which plasmid pBS-CrtH for the expression of the ORF of *CrtH* was co-transformed with plasmid pACCAR16ΔcrtX, which was genetically engineered to express genes required for the biosynthesis of ß-carotene (Misawa *et al*., 1995, Biochem Biophys Res Commun 209:867-876). HPLC profiles showed that *E. coli* cells harboring plasmids pBS-CrtH and pACCAR16ΔcrtX produced ß-carotene, ß-cryptoxanthin and zeaxanthin (**Fig. 4**). Of total detectable carotenoids , ß-cryptoxanthin accounted for 24.5% (±0.4%) and zeaxanthin accounted for 38.9% (±0.7%). As expected, the negative control *E. coli* strain harboring plasmids pBluescriptII KS (+) and pACCAR16ΔcrtX accumulated only ß-carotene.

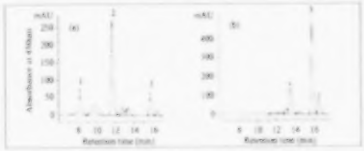


Fig. 4 HPLC profiles of carotenoids extracted from *E. coli* cells carrying plasmids (a) pBS-CrtH and pACCAR16ΔcrtX, (b) empty cloning vector pBluescriptII KS (+) and pACCAR16ΔcrtX. Peaks numbered 1, 2 and 3 correspond to zeaxanthin, ß-cryptoxanthin and ß-carotene, respectively.

Expression of *CrtH* in *A. thaliana* leads to the accumulation of violaxanthin

To investigate the biochemical function of *CrtH* in planta, a plant transformation construct for overexpressing this gene under the control of the seed-specific promoter napin was made, and used to transform *A. thaliana* wild type (wt) and b1b2 mutant that has T-DNA knockouts in the two ß-carotene hydroxylase genes (Tian *et al*., 2003, Plant Cell 15:1320-1332). Northern blot analysis (**Fig. 5**) showed that all transgenic plants selected for analysis had significant levels of *CrtH* expression in immature siliques, while no expression could be detected in untransformed control plants. HPLC analysis (**Fig. 6**) showed 2%-40% decrease in the amount of ß-carotene in most of the transgenic lines, 3%-160% increase in lutein, at least 3-fold increase in violaxanthin, and 4%-240% increase in zeaxanthin.

Fig. 5 Northern analysis of *CrtH* in immature siliques of transgenic *Arabidopsis thaliana*. (a) wild type overexpressing *CrtH* (BY275-BY284), (b) b1b2 double mutant overexpressing *CrtH* (BY317-BY347).

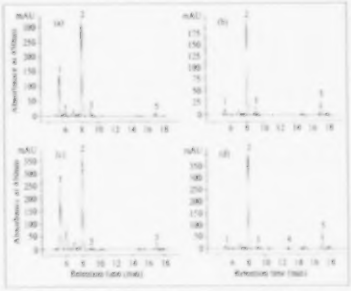


Fig. 6 HPLC profiles of carotenoid extracted from seeds of *Arabidopsis thaliana*. (a) wild type overexpressing *CrtH*, (b) wild type, (c) b1b2 mutant overexpressing *CrtH* and (d) b1b2 mutant. Peaks numbered 1, 2, 3, 4 and 5 correspond to violaxanthin, lutein, zeaxanthin, ß-cryptoxanthin and ß-carotene, respectively.

Conclusion

We have cloned the full-length cDNA sequence of ß-carotene hydroxylase from *Adonis aestivalis*. Functional assays in *E. coli* showed CrtH to have ß-carotene hydroxylase activity, which converted ß-carotene to ß-cryptoxanthin and zeaxanthin. Further biochemical and molecular characterization of this protein in *Arabidopsis* wild type and b1b2 mutant having T-DNA knock-outs in the two ß-carotene hydroxylase genes revealed that *CrtH* overexpression resulted in a marked increase in levels of violaxanthin. This gene is an additional tool that will allow for further understanding, dissection, and modification of the bio-synthetic pathway of carotenoids in higher plants.

Canada

Agriculture and Agri-Food Canada | Agriculture et Agroalimentaire Canada

Enhancing carotenoid content of *Brassica napus* seeds by downregulating lycopene epsilon cyclase

Bianyun Yu*, Derek Lydiate, Ulrike Schäfer, and Abdelali Hannoufa
Agriculture and Agri-Food Canada, Research Centre, 107 Science Place,
Saskatoon, Saskatchewan, Canada S7N 0X2 *yub@agr.gc.ca

The accumulation of carotenoids in higher plants is regulated by the environment, tissue type and developmental stage. The carotenoid biosynthesis pathway branches after the formation of the lycopene to form carotenoids with either two β-rings or one β- and one ε-ring (Fig. 1). We used RNAi constructs of lycopene ε-cyclase (*ε-CYC*) to alter carotenoid accumulation in seeds of *B. napus*. Transgenic seeds expressing these constructs had increased levels of β-carotene, zeaxanthin, violaxanthin and, unexpectedly, lutein. The higher total carotenoid content in *ε-CYC* silenced seeds suggests that this gene is a rate-limiting step in the carotenoid biosynthesis pathway.

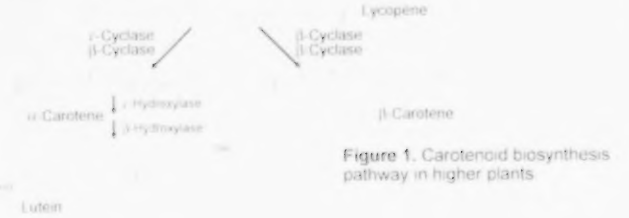


Figure 1. Carotenoid biosynthesis pathway in higher plants

Carotenoid profiles of developing seeds of *B. napus*

The profiles of carotenoids accumulating in the seeds varied depending on the developmental stage. The highest level of violaxanthin was detected in seeds 15-20 days post anthesis (dpa) and it gradually decreased as the seeds matured. Seeds 35-40 dpa had the highest levels of lutein and β-carotene, with much lower concentrations observed in fresh mature and dry mature seeds (Fig. 2).

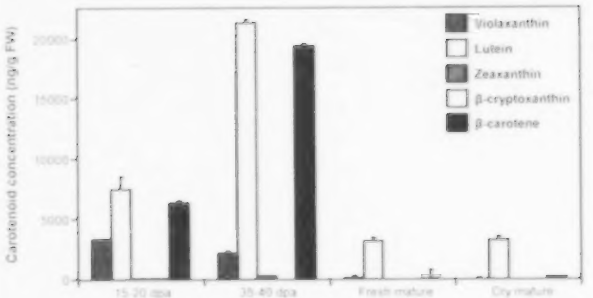


Figure 2. Concentrations of carotenoid compounds in developing seeds of *Brassica napus* DH12075

Silencing of *ε-CYC* increased levels of β-carotene and lutein

Two RNAi constructs, 710-422 and 710-423 (Fig. 3A) were made to the 5' and 3' ends, respectively, of *B. napus ε-CYC* and used to transform *B. napus* DH12075 line. Only the expression of *ε-CYC* was reduced in transgenic lines relative to the untransformed control (Fig. 3B). Expression of the other genes was unaltered by the *ε-CYC* RNAi.

Visual observation of the color of carotenoid extracts from the mature seeds of *ε-CYC* silenced lines and the untransformed control (Fig. 4) combined with HPLC analysis showed seeds of all transgenic lines had enhanced concentrations of total carotenoids (Table 1). The ratio of β-carotene to lutein approximately doubled in the seeds of most transgenic lines, although 4.8, 4.9 and 8 fold in the relative amounts of β-carotene to lutein were observed in lines BY223, BY365 and BY269, respectively.

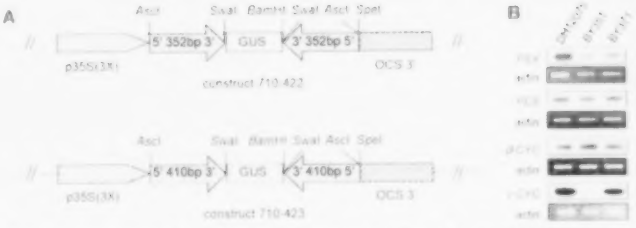


Figure 3. [A] Diagram of RNAi constructs 710-422 and 710-423. **[B]** Gene expression in the developing seeds of select *ε-CYC* RNAi lines. *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *β-CYC*, lycopene β-cyclase; *ε-CYC*, lycopene ε-cyclase

Figure 4. Carotenoid extracts from dry mature seeds of *ε-CYC* RNAi lines BY223, BY365 and BY54 and untransformed control DH12075

Table 1. Concentrations of carotenoid compounds in seeds of *B. napus ε-CYC* RNAi lines and untransformed control DH12075

Plant line	β-carotene µg/g FW	Lutein µg/g FW	Violaxanthin µg/g FW	Zeaxanthin µg/g FW	β-cryptoxanthin µg/g FW	Total carotenoid µg/g FW	Ratio of β-carotene to lutein
DH12075	0.49 ± 0.28	3.30 ± 0.49	UD	UD	UD	5.34 ± 0.96	0.15
Construct 710-422							
BY173	15.07 ± 4.98	39.10 ± 5.33	2.43 ± 0.18	0.44 ± 0.13	0.23 ± 0.04	75.45 ± 11.23	0.39
BY228	19.52 ± 3.27	41.74 ± 3.80	1.30 ± 0.11	2.51 ± 0.69	0.08 ± 0.13	86.59 ± 8.57	0.47
BY241	4.18 ± 0.29	14.05 ± 0.21	0.61 ± 0.02	0.21 ± 0.02	0.10 ± 0.07	24.31 ± 0.56	0.30
BY269	90.76 ± 6.72	76.22 ± 3.74	1.74 ± 0.07	7.07 ± 0.13	0.30 ± 0.13	227.78 ± 2.85	1.19
BY351	6.56 ± 4.14	17.44 ± 3.46	1.09 ± 0.12	0.14 ± 0.03	UD	34.44 ± 11.22	0.36
Construct 710-423							
BY54	11.09 ± 2.06	23.15 ± 6.64	0.91 ± 0.24	0.35 ± 0.03	0.16 ± 0.06	46.95 ± 8.80	0.48
BY58	3.34 ± 1.67	9.46 ± 3.05	0.36 ± 0.13	0.33 ± 0.22	UD	19.07 ± 5.82	0.35
BY223	17.68 ± 1.25	25.29 ± 1.16	1.48 ± 0.12	0.31 ± 0.05	0.15 ± 0.01	59.81 ± 3.19	0.71
BY365	27.02 ± 4.04	37.64 ± 3.95	2.42 ± 0.25	1.13 ± 0.33	0.15 ± 0.03	94.09 ± 12.57	0.72
BY371	3.50 ± 0.31	10.36 ± 0.57	0.45 ± 0.07	0.28 ± 0.01	UD	19.15 ± 2.48	0.34

UD: undetectable; FW: fresh weight. Each value is the mean result from triplicate ± SD

ε-CYC was reported to play a key role in controlling the ration of lutein to β,β-carotenoids (Pogson et al., 1996, Plant Cell, 8: 162). We used RNAi constructs to repress *ε-CYC* expression in *B. napus*. Of the carotenoid biosynthesis genes examined, only *ε-CYC* transcription appeared to be affected by expression of the RNAi construct, yet these plants had major alterations in their carotenoid profiles. This suggests that the accumulation of carotenoids in transgenic seeds was not due to enhanced *de novo* biosynthesis. The higher concentration of carotenoids in the transgenic plants may be due to a greater flux of substrate through the pathway or alteration in plastid morphology that resulted in increased capacity for carotenoid storage.

Major increase in the carotenoid content of *Brassica* oilseeds could be achieved by knocking down *ε-CYC*, which appears to control a rate limiting step in carotenoid biosynthesis.

We are grateful to Delwin Epp for technical assistance. This work was supported by a grant from Saskatchewan Agriculture Development Fund.

© 2007

Canada

Agriculture and Agri-Food Canada | Agriculture et Agroalimentaire Canada

Enhancing carotenoid content of *Brassica napus* seeds by downregulating lycopene epsilon cyclase

Bianyun Yu*, Derek Lydiate, Ulrike Schäfer, and Abdelali Hannoufa
Agriculture and Agri-Food Canada, Research Centre, 107 Science Place,
Saskatoon, Saskatchewan, Canada S7N 0X2 *yub@agr.gc.ca

Summary

The accumulation of carotenoids in higher plants is regulated by the environment, tissue type and developmental stage. The carotenoid biosynthesis pathway branches after the formation of the lycopene to form carotenoids with either two β-rings or one β- and one ε-ring (Fig. 1). We used RNAi constructs of lycopene ε-cyclase (ε-*CYC*) to alter carotenoid accumulation in seeds of *B. napus*. Transgenic seeds expressing these constructs had increased levels of β-carotene, zeaxanthin, violaxanthin and, unexpectedly, lutein. The higher total carotenoid content in ε-*CYC* silenced seeds suggests that this gene is a rate-limiting step in the carotenoid biosynthesis pathway.

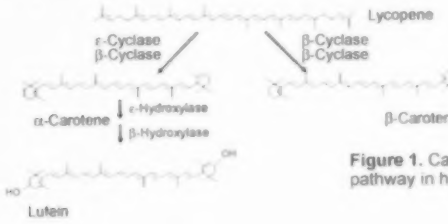


Figure 1. Carotenoid biosynthesis pathway in higher plants.

Results

Carotenoid profiles of developing seeds of *B. napus*

The profiles of carotenoids accumulating in the seeds varied depending on the developmental stage. The highest level of violaxanthin was detected in seeds 15-20 days post anthesis (dpa) and it gradually decreased as the seeds matured. Seeds 35-40 dpa had the highest levels of lutein and β-carotene, with much lower concentrations observed in fresh mature and dry mature seeds (Fig. 2).

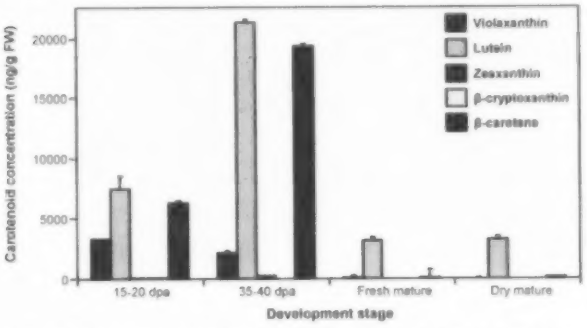


Figure 2. Concentrations of carotenoid compounds in developing seeds of *Brassica napus* DH12075.

Silencing of ε-*CYC* increased levels of β-carotene and lutein

Two RNAi constructs, 710-422 and 710-423 (Fig. 3A) were made to the 5' and 3' ends, respectively, of *B. napus* ε-*CYC* and used to transform *B. napus* DH12075 line. Only the expression of ε-*CYC* was reduced in transgenic lines relative to the untransformed control (Fig. 3B). Expression of the other genes was unaltered by the ε-*CYC* RNAi.

Visual observation of the color of carotenoid extracts from the mature seeds of ε-*CYC* silenced lines and the untransformed control (Fig. 4) combined with HPLC analysis showed seeds of all transgenic lines had enhanced concentrations of total carotenoids (Table 1). The ratio of β-carotene to lutein approximately doubled in the seeds of most transgenic lines, although 4.8, 4.9 and 8 fold in the relative amounts of β-carotene to lutein were observed in lines BY223, BY365 and BY269, respectively.

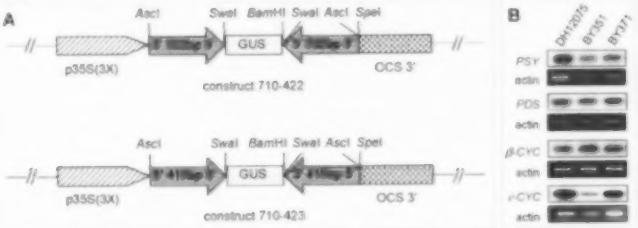


Figure 3. [A] Diagram of RNAi constructs 710-422 and 710-423. **[B]** Gene expression in the developing seeds of select ε-*CYC* RNAi lines. *PSY*, phytoene synthase; *PDS*, phytoene desaturase; β-*CYC*, lycopene β-cyclase, ε-*CYC*, lycopene ε-cyclase.

Figure 4. Carotenoid extracts from dry mature seeds of ε-CYC RNAi lines BY223, BY365 and BY54 and untransformed control DH12075.

Table 1. Concentrations of carotenoid compounds in seeds of *B. napus* ε-*CYC* RNAi lines and untransformed control DH12075.

Plant line	β-carotene µg/g FW	Lutein µg/g FW	Violaxanthin µg/g FW	Zeaxanthin µg/g FW	β-cryptoxanthin µg/g FW	Total carotenoid µg/g FW	Ratio of β-carotene to lutein
DH12075	0.49 ± 0.28	3.30 ± 0.49	UD	UD	UD	9.34 ± 0.96	0.15
Construct 710-422							
BY173	15.07 ± 4.90	39.10 ± 5.33	2.43 ± 0.18	0.44 ± 0.13	0.23 ± 0.04	75.45 ± 11.23	0.39
BY228	19.52 ± 3.27	41.74 ± 3.00	1.30 ± 0.11	2.51 ± 0.68	0.08 ± 0.13	86.59 ± 8.57	0.47
BY241	4.18 ± 0.28	14.05 ± 0.21	0.61 ± 0.02	0.21 ± 0.02	0.10 ± 0.07	24.31 ± 0.56	0.30
BY269	90.76 ± 6.72	76.22 ± 3.74	1.74 ± 0.07	7.07 ± 0.13	0.30 ± 0.13	227.75 ± 2.85	1.19
BY351	6.36 ± 4.14	17.44 ± 3.46	1.08 ± 0.32	0.14 ± 0.03	UD	34.44 ± 11.22	0.36
Construct 710-423							
BY54	11.09 ± 2.06	23.15 ± 0.64	0.91 ± 0.24	0.35 ± 0.03	0.16 ± 0.08	46.95 ± 8.00	0.48
BY58	3.34 ± 1.87	9.46 ± 3.00	0.38 ± 0.13	0.33 ± 0.22	UD	19.07 ± 5.82	0.35
BY223	17.88 ± 1.25	25.29 ± 1.16	1.46 ± 0.12	0.31 ± 0.05	0.15 ± 0.01	50.81 ± 3.19	0.71
BY365	27.02 ± 4.04	37.64 ± 3.95	2.42 ± 0.25	1.73 ± 0.33	0.15 ± 0.03	94.09 ± 12.07	0.72
BY371	3.50 ± 0.31	10.36 ± 0.57	0.45 ± 0.07	0.28 ± 0.01	UD	19.15 ± 2.48	0.34

UD: undetectable, FW: fresh weight. Each value is the mean result from triplicate ± SD

Discussion

ε-*CYC* was reported to play a key role in controlling the ration of lutein to β,β-carotenoids (Pogson et al., 1996, Plant Cell, 8: 162). We used RNAi constructs to repress ε-*CYC* expression in *B. napus*. Of the carotenoid biosynthesis genes examined, only ε-*CYC* transcription appeared to be affected by expression of the RNAi construct, yet these plants had major alterations in their carotenoid profiles. This suggests that the accumulation of carotenoids in transgenic seeds was not due to enhanced *de novo* biosynthesis. The higher concentration of carotenoids in the transgenic plants may be due to a greater flux of substrate through the pathway or alteration in plastid morphology that resulted in increased capacity for carotenoid storage.

Conclusion

Major increase in the carotenoid content of *Brassica* oilseeds could be achieved by knocking down ε-*CYC*, which appears to control a rate limiting step in carotenoid biosynthesis.

Acknowledgments

We are grateful to Delwin Epp for technical assistance. This work was supported by a grant from Saskatchewan Agriculture Development Fund.

© 2007